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BLOOD

The Journal of The American Society of Hematology

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[1]Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.

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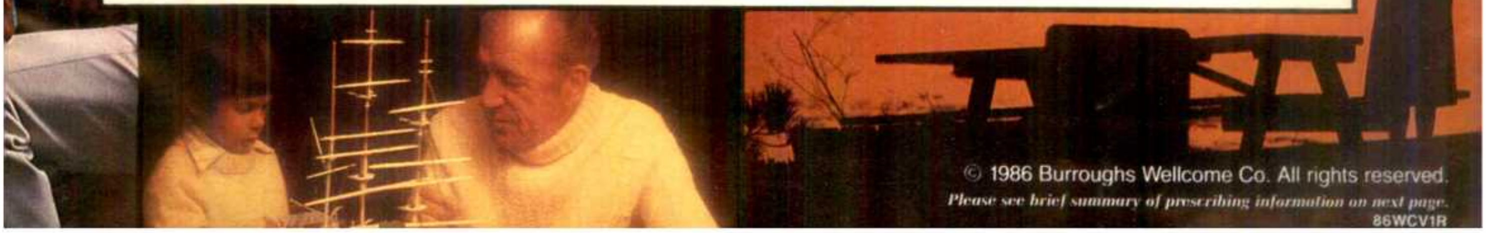

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Please see brief summary of prescribing information on next page.

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Before prescribing WELLCOVORIN® Tablets, please consult complete prescribing information. The following is a brief summary.

INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer*, *41*(1):36-51, 1978.
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5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis*, *135*(3): 493-496, 1977.

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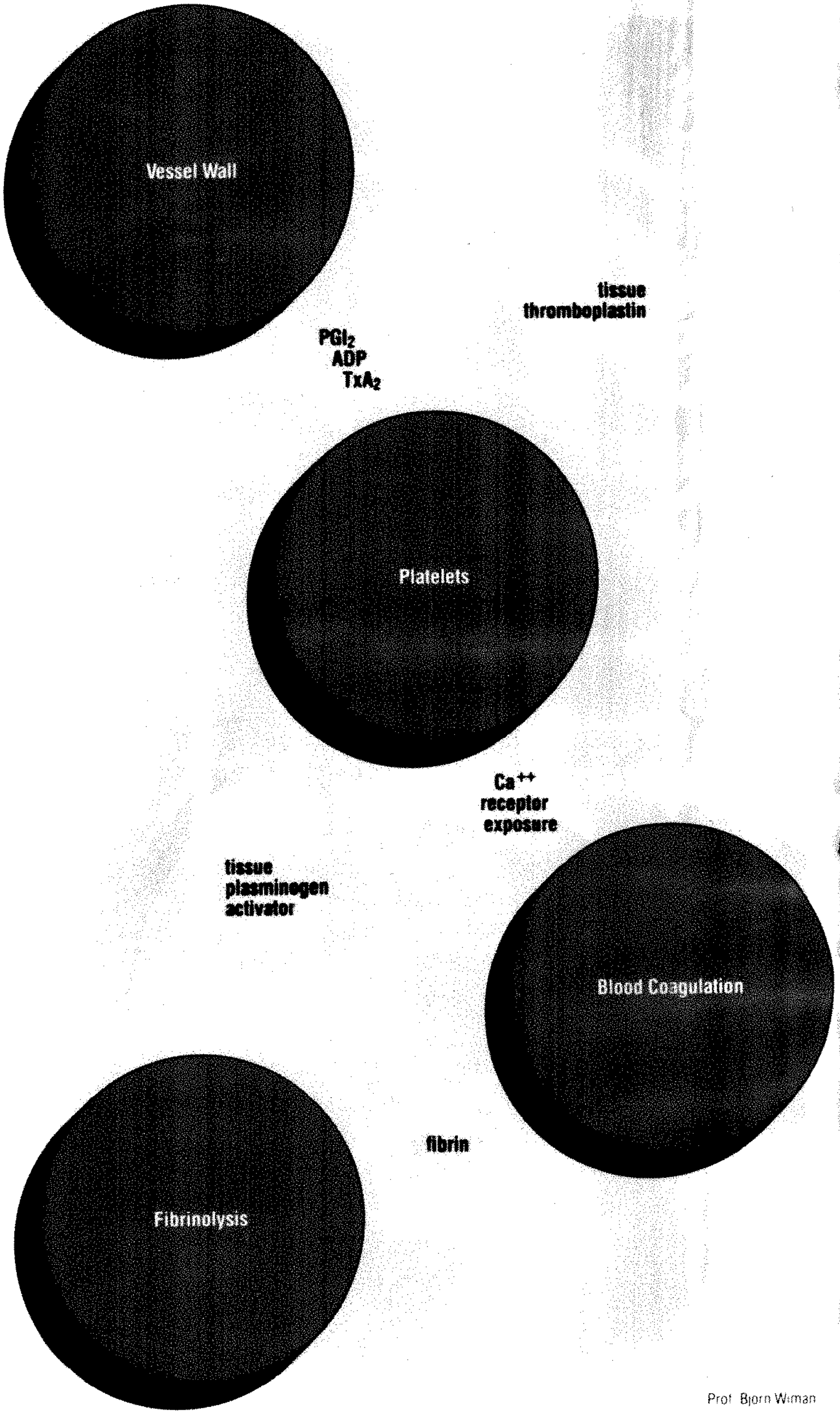

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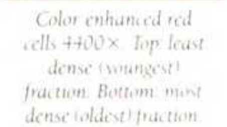

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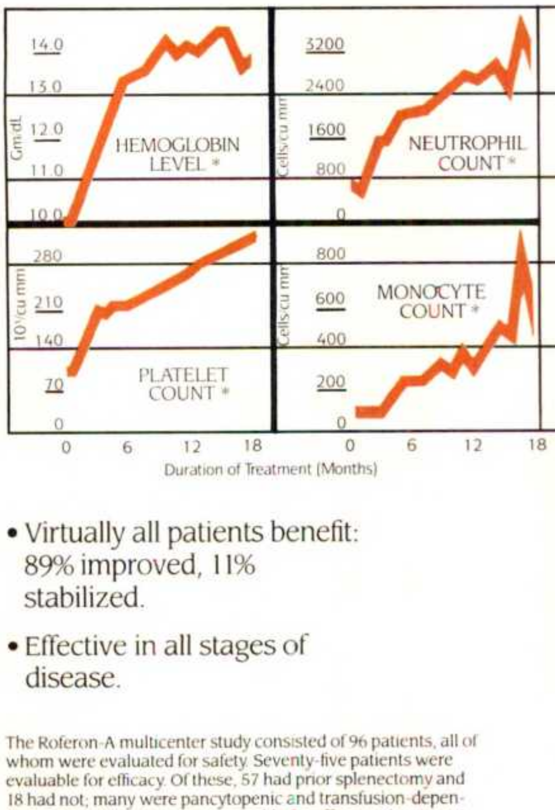

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*Multicenter Study, Data on file, Hoffmann-La Roche Inc., Nutley, NJ.

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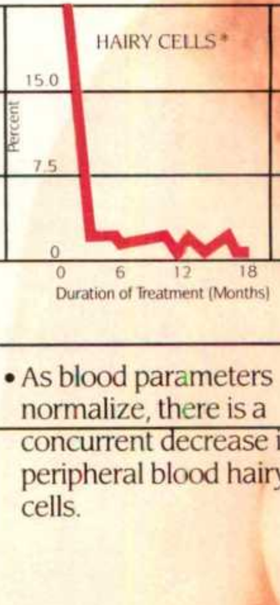

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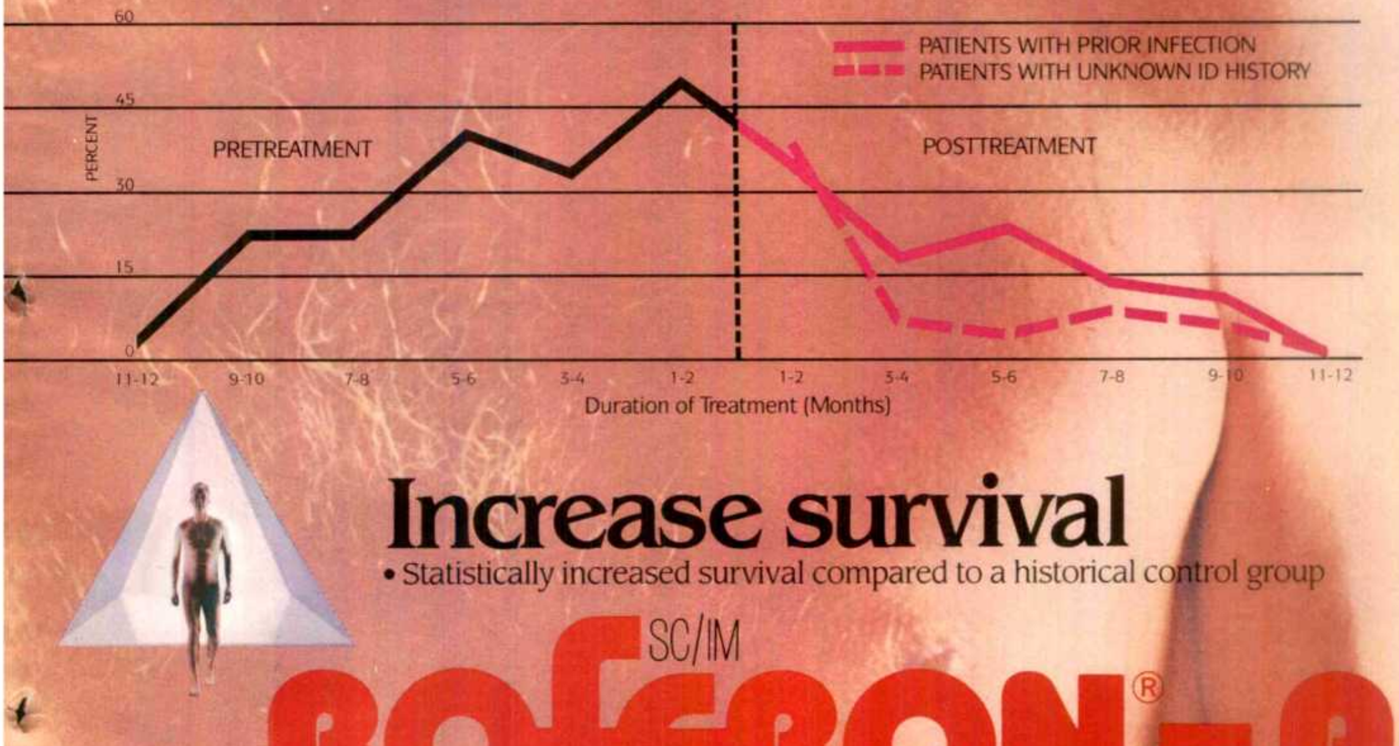

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PRECAUTIONS: *General:* When considering for chemotherapy, evaluate need and usefulness of the drug against risk of adverse reactions. Most adverse reactions are reversible if detected early. If severe reactions occur, reduce dosage or discontinue drug and take appropriate corrective measures. Reinstitute therapy with caution and with adequate consideration of the further need for the drug and alertness to possible recurrence of toxicity.

The minimum effective dose in hairy cell leukemia not established.

Information for Patient: Caution patients not to change brands of Interferon without medical consultation, as a change in dosage may result. Inform patients regarding potential benefits and risks. If home use is desirable, give instructions on appropriate use, including review of the Patient Information Sheet. Patients should be well hydrated, especially during initial stages of treatment.

Laboratory Tests: Complete blood counts and liver function tests should be performed prior to therapy and at appropriate periods during therapy. Since responses are not generally observed for one to three months after treatment initiation, very careful monitoring for severe depression of blood cell counts is warranted during the initial phase.

Patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during treatment.

Carcinogenesis, Mutagenesis and Impairment of Fertility: Carcinogenesis: Roferon-A has not been tested for carcinogenic potential.

Mutagenesis: A. Internal studies. Ames test results revealed no evidence of mutagenicity. Human lymphocyte cultures were treated *in vitro* at noncytotoxic concentrations. No increase in chromosomal damage noted. B. Published studies. No published studies on mutagenic potential. However, a number of studies on the genotoxicity of human leukocyte interferon have been reported. A chromosomal defect has been reported after the addition of human leukocyte interferon to lymphocyte cultures from a patient suffering from a lymphoproliferative disorder. Other studies have failed to detect chromosomal abnormalities following treatment of lymphocyte cultures from healthy volunteers with human leukocyte interferon. Protection from chromosomal aberrations produced by gamma rays observed in primary chick embryo fibroblasts.

Impairment of Fertility: Nonpregnant female rhesus monkeys treated at doses of 5 and 25 million IU/kg/day have shown menstrual cycle irregularities, including prolonged or shortened menstrual periods and erratic bleeding; these cycles were considered to be anovulatory. Monkeys returned to a normal menstrual rhythm following discontinuation of treatment.

Drug Interactions: Not fully evaluated.

PREGNANCY: Pregnancy Category C. Safe use in human pregnancy not established. Therefore, use during pregnancy only if potential benefit justifies potential risk to fetus. Primate studies showed dose-related menstrual irregularities and an increased incidence of spontaneous abortions. Decreases in serum estradiol and progesterone concentrations reported in women treated with human leukocyte interferon. Therefore, fertile women should not receive Roferon-A unless they are using effective contraception during therapy. Male fertility and teratologic evaluations have yielded no significant adverse effects.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants, a decision should be made whether to discontinue nursing or the drug, taking into account the importance of the drug to the mother.

Pediatric Use: Safety and effectiveness in children under 18 not established.

ADVERSE REACTIONS: The following data on adverse reactions are based on the SC or IM administration of Roferon-A as a single agent for the treatment of 63 patients with hairy cell leukemia during investigational trials in the U.S.

Flu-like syndromes consisting of fatigue (89%), fever (98%), chills (64%), myalgias (73%), headache (71%) occurred in the majority of patients and tended to diminish with continuing therapy. Other side effects such as anorexia (46%), nausea (32%), emesis (10%), diarrhea (29%), dizziness (21%), rash (18%), change in taste (13%), dryness or inflammation of the oropharynx (16%), dry skin or pruritus (13%), weight loss (14%) were observed with moderate frequency. Less commonly, diaphoresis (8%), paresthesias (6%), numbness (6%), partial alopecia (8%), reactivation of herpes labialis (8%), transient impotence (6%), arthralgias (5%) were also observed. Rarely (<3%), CNS effects including decreased mental status, depression, visual disturbances, sleep disturbances, nervousness, as well as cardiac adverse events, including hypertension, chest pain, arrhythmias, palpitations, were reported. Adverse experiences which occurred rarely and may have been related to underlying disease included epistaxis, bleeding gums, ecchymosis, petechiae. Miscellaneous adverse events, such as night sweats, urticaria, conjunctivitis, inflammation at the site of injection, were also rarely observed.

Roferon-A has also been evaluated for the treatment of many other types of cancer under investigation in the U.S. These studies generally utilized higher doses (12 to 50 million $\mathrm{IU/m^2}$). All of the above adverse reactions which occurred in patients with hairy cell leukemia were also observed in patients receiving higher doses. The incidence of most adverse reactions was similar between the two groups, but tended to be more severe in the higher dosage group. Additional adverse effects which occurred in these patients included confusion (10%), hypotension (6%), lethargy (3%), edema (3%). Adverse experiences occurring in less than 1% of these patients and observed only in patients with malignancies other than hairy cell leukemia are as follows: *GI:* abdominal fullness, hypermotility, hepatitis; *CNS:* gait disturbance, poor coordination, hallucinations, syncope, seizures, encephalopathy, psychomotor retardation, coma, stroke, transient ischemic attacks, aphasia, aphonia, dysarthria, dysphasia, forgetfulness, amnesia, sedation, apathy, anxiety, emotional lability, irritability, hyperactivity, involuntary movements, claustrophobia, loss of libido; *Peripheral Nervous System:* muscle contractions; *Cardiovascular:* congestive heart failure, pulmonary edema, myocardial infarction, Raynaud's phenomenon, hot flashes; *Pulmonary:* bronchospasm, tachypnea; *Miscellaneous:* excessive salivation, flushing of skin.

Abnormal Laboratory Test Values. The percentages of abnormal laboratory test values seen in 1019 patients evaluated in overall safety trials (including hairy cell leukemia patients) are: *Hematologic:* leukopenia (69%), neutropenia (58%), thrombocytopenia (42%), decreased hemoglobin (6.3%), decreased hematocrit (12.5%); mean time to nadir: WBC—22 days, platelets—17 days. *Hepatic:* SGOT (78%), alkaline phosphatase (48%), LDH (47%), bilirubin (31%). *Renal/Urinary:* BUN (10%), serum creatinine (10%), uric acid (15%), proteinuria (25%). *Other Tests:* hypocalcemia (51%), elevated serum glucose (39%), elevated serum phosphorus (17%).

Neutralizing antibodies were detected in approximately 27% of all patients (3.4% for hairy cell leukemia patients). No clinical sequelae of their presence have been documented. Antibodies may occur spontaneously in certain clinical conditions.

DOSAGE AND ADMINISTRATION: Hairy Cell Leukemia—The dose of Roferon-A is 3 million IU daily for 16 to 24 weeks, administered as an SC or IM injection. SC administration is particularly advisable for, but not limited to, thrombocytopenic patients (platelet count <50,000) or for patients at risk for bleeding. The recommended maintenance dose is 3 million IU, 3 times/week. Dosage reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur. The use of doses higher than 3 million IU is not recommended. Treatment should be continued for 6 months before determining whether to discontinue therapy in nonresponding patients. The optimal duration of treatment has not been determined in responding patients. If severe reactions occur, modify dosage (50% reduction) or temporarily discontinue therapy until adverse reactions abate. The need for dosage reduction should take into account the effects of prior X-ray therapy or chemotherapy that may have compromised bone marrow reserve.

HOW SUPPLIED: Roferon-A Injectable Solution: Vials containing 3 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/mL). Boxes of 10 (NDC 0004-1987-01). Roferon-A Injectable Solution: Vials containing 18 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/0.5 mL). Boxes of 1 (NDC 0004-1988-09). Roferon-A Sterile Powder for Injection: Vials containing 18 million IU Interferon alfa-2a, recombinant/Roche, with accompanying diluent. Boxes of 1 (NDC 0004-1993-09). For reconstitution, storage and handling directions, see complete product information.

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Common side effects usually self-limiting and manageable

Initial side effects, such as fever, chills, myalgias, headache and fatigue, are self-limiting and generally manageable. Myelosuppression, which can occur early in the treatment course, warrants close clinical and laboratory observation and is usually alleviated by dose reduction or temporary discontinuation of drug. Roferon-A should be used with caution in patients with severe preexisting cardiac disease, severe renal or hepatic disease, seizure disorders and/or compromised central nervous system function. Persistent fatigue is found to be less troublesome with *p.m.* or *h.s.* administration.

SC/IM fixed dosing simple enough for self-administration

Ready-to-Use Injectable Solution

No mixing or reconstitution necessary

3 million IU vial (3 million IU/mL)

18 million IU vial (3 million IU/0.5 mL)

Sterile Powder for Injection

18 million IU vial (3 million IU/0.5 mL)

3 mL diluent for reconstitution

Must be refrigerated at temperatures of 36° to 46°F (2° to 8°C). At no time should Roferon-A Ready-to-Use Injectable Solution remain unrefrigerated for more than 24 hours. The Sterile Powder for Injection should not remain unrefrigerated for more than 48 hours. Once the powder is reconstituted, it should be refrigerated and must be used within 30 days. Do not freeze or shake.

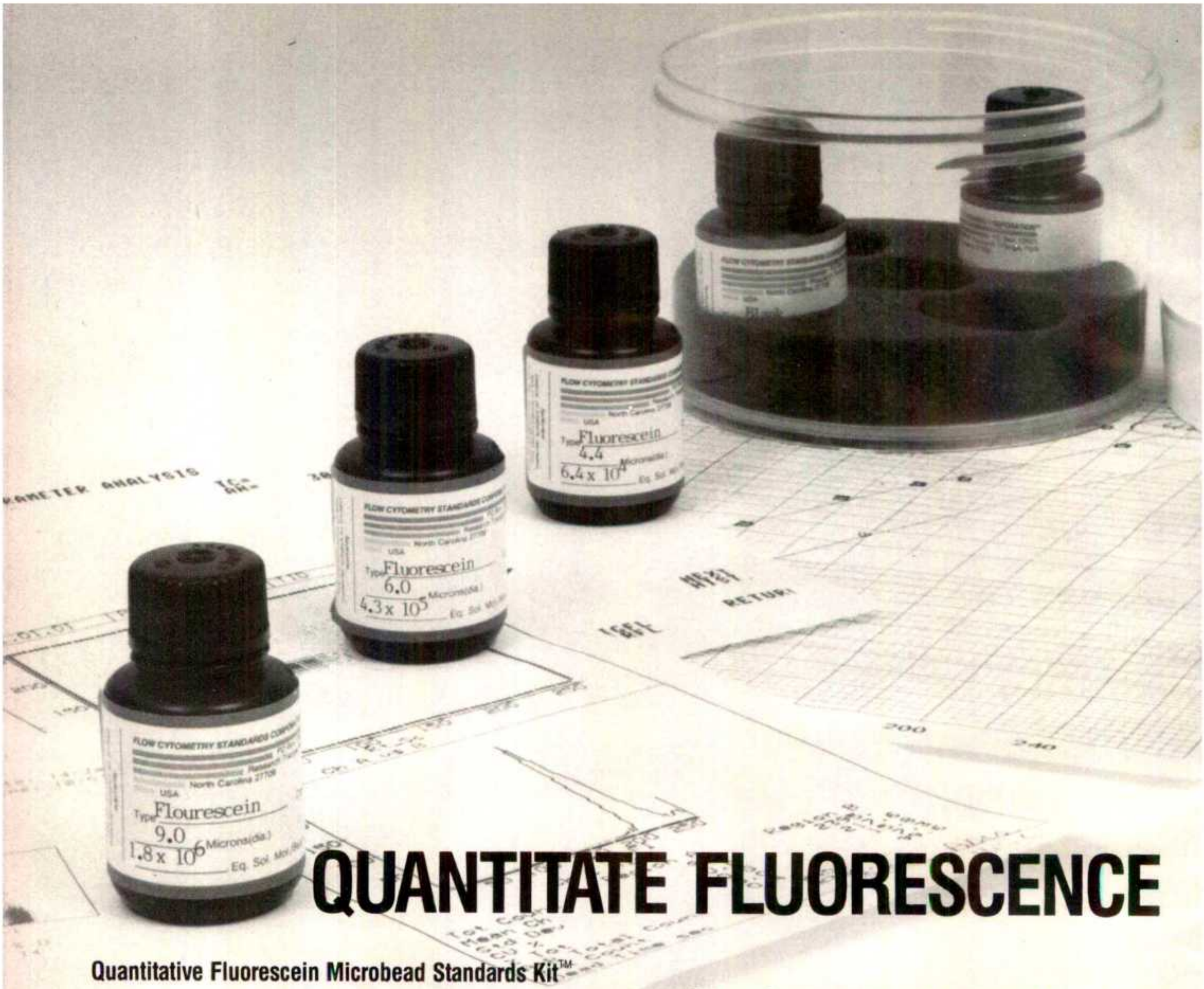
SC/IM

ROFERON®-A

BRAND OF Interferon alfa-2a, recombinant/Roche

The fourth modality

Please see summary of product information on adjacent page.



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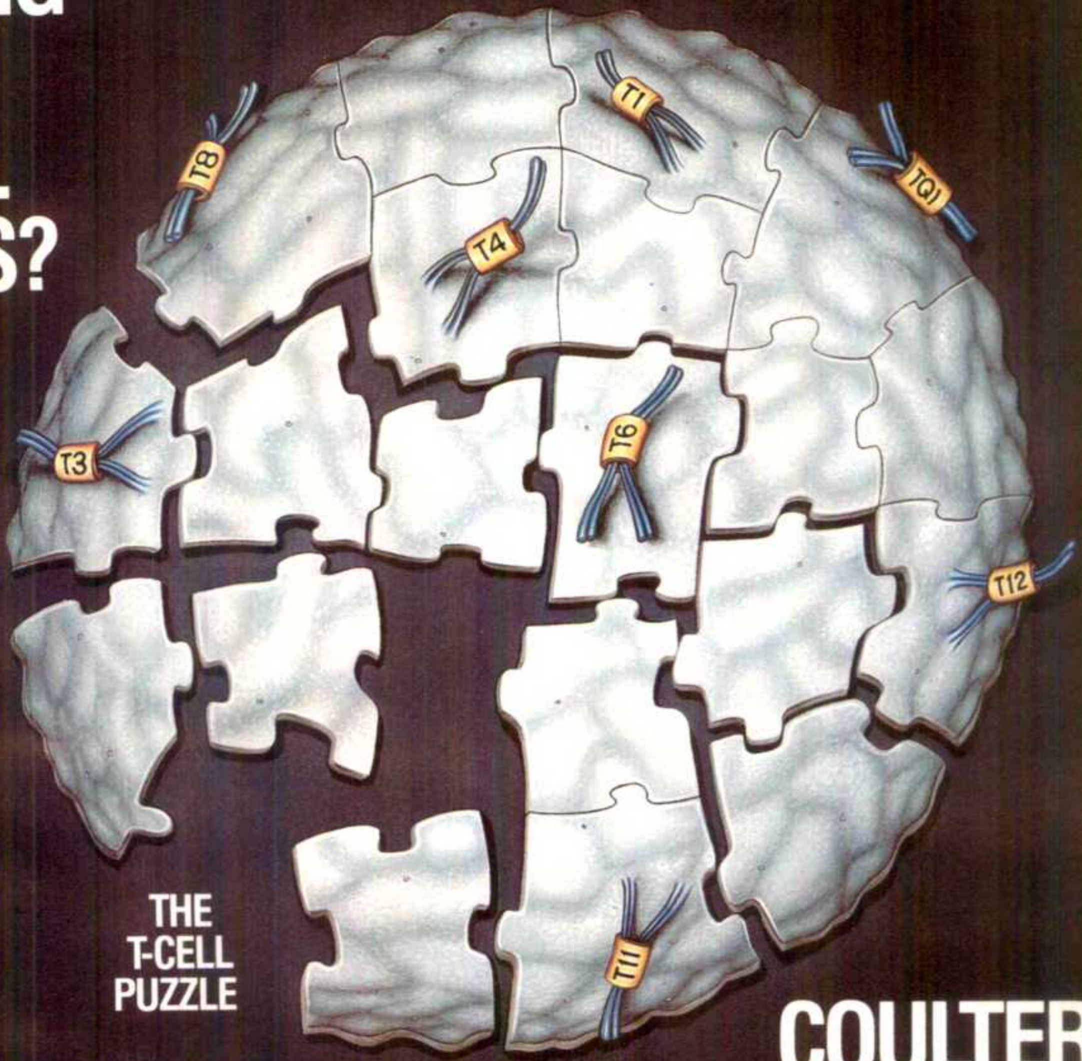

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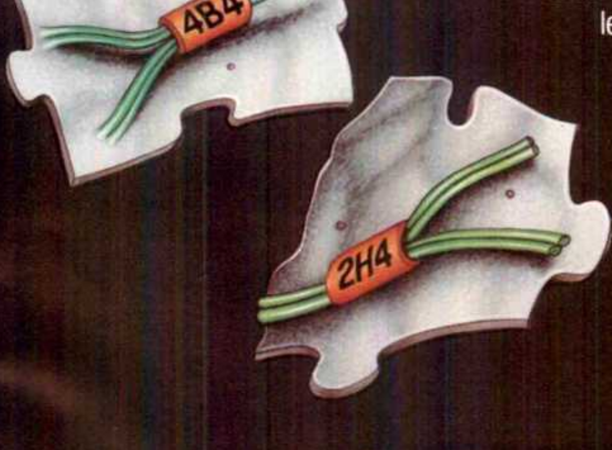

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1. In bath or shower. Fingers flat, move opposite hand gently over each breast. Check for lumps, hard knots, thickening.

2. In front of a mirror. Observe breasts. Arms at sides. Raise arms high overhead. Any change in nipples, contours, swelling, dimpling of skin? Palms on hips: press down firmly to flex chest muscles. Breasts do not usually match.

3. Lying down. Pillow under right shoulder, right hand behind head. Left hand fingers flat, press gently in small circular motions starting at 12 o'clock. Make about three circles moving closer to and including nipple. Reverse and repeat on left.

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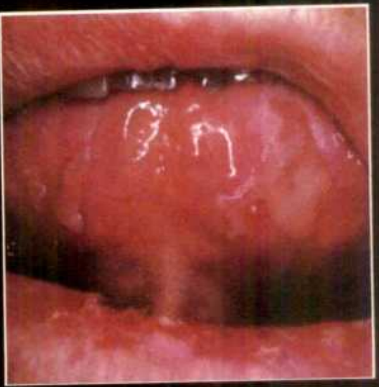
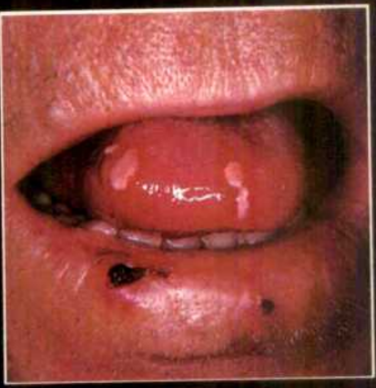
Radiogenic mucositis or herpes simplex?

See answer under flap.

The diagnostic challenge in immunocompromised patients...





Early intervention can prevent or reduce HSV complications

A high percentage of immunocompromised patients are at risk for developing HSV infections. Timely therapy with an antiviral agent can avoid the incidence of secondary complications, such as visceral dissemination, and prolonged hospital stay.

Appearances may be deceiving

Both slides are herpes simplex. In immunocompromised patients, mucositis and stomatitis that appear to result from radiation therapy or immunosuppression may actually be caused by herpes simplex. Differential diagnosis can be problematic, but appropriate laboratory tests can aid in the determination.

There is an effective therapeutic agent available

ZOVIRAX I.V. stops viral replication, speeds healing, and reduces pain. It is effective antiviral therapy for treatment of herpes simplex viral infections.

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(acyclovir sodium)
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When the problem begins with a virus, end it with an antiviral.

Please see following page for brief summary of prescribing information.

ZOVIRAX® I.V.
(acyclovir sodium)
STERILE POWDER / I.V. INFUSION

ZOVIRAX® I.V. INFUSION (acyclovir sodium) sterile powder

FOR INTRAVENOUS INFUSION ONLY

INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) sterile powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: **1.** C.D. Mitchell, *et al.*, Lancet *I*(8235): 1389-1392, Jun. 27, 1981. **2.** Wade, *et al.*, Ann. Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am J. Med. *73*(1A): 229-235, Jul. 20, 1982. **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *I*(8274): 697-700, Mar. 27, 1982. **6.** Z.M. Naib, *et al.*, Cancer Res. *33*:1452-1463, 1973.

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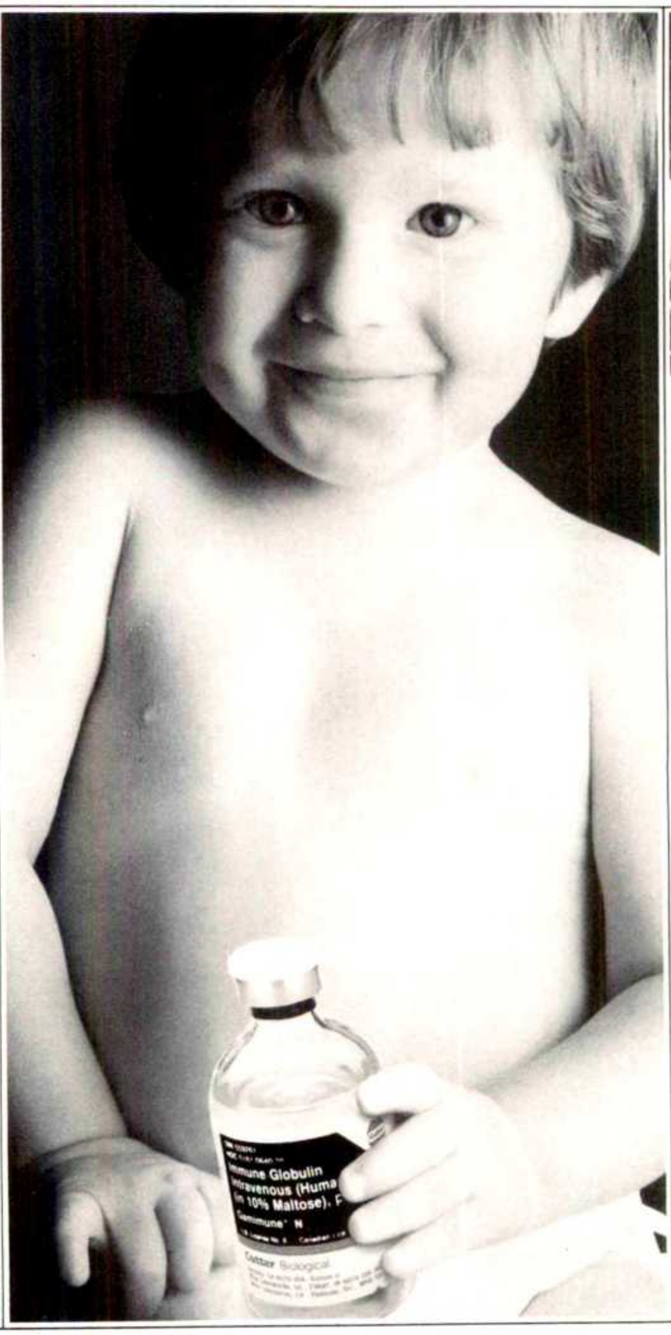
GAMIMUNE® N may initiate a therapeutic increase in platelets in both children and adults with idiopathic thrombocytopenic purpura (ITP).[1*†] Treatment with GAMIMUNE® N has enabled some ITP patients to undergo major surgery.[1]



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Cutter's fractionation method (Cohn-Oncley 6 & 9) contains all the necessary steps to produce high purity immune globulin. GAMIMUNE® N contains no foreign contaminants, no detectable levels of protein aggregates or vasoactive enzymes.[2] With GAMIMUNE® N you get what you expect …IGIV containing at least 98% pure gamma globulin.[1]

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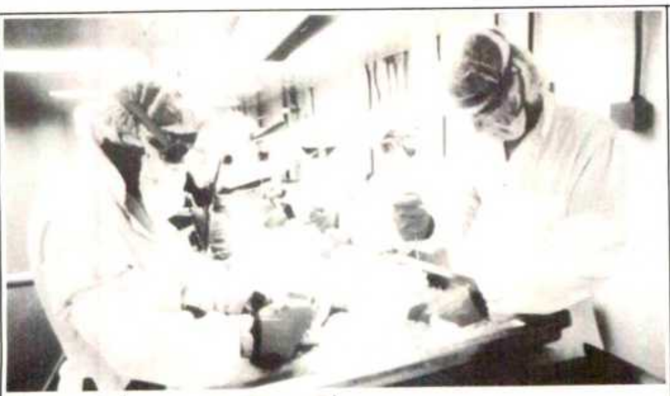

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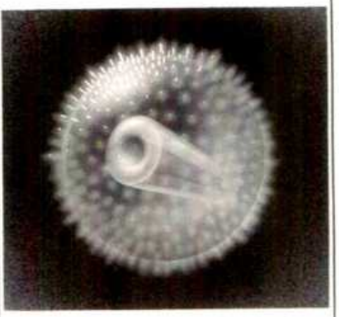
[‡] The Cohn-Oncley process used to produce Gamimune®N has been challenged *in vitro* with a virus spike of 1 x 10^5 TCID (Tissue Culture Infectious Doses) of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, Gamimune®N undergoes an incubation step as a final container which has been shown to effect an additional 1 x 10^3 to 1 x 10^4 TCID (Tissue Culture Infectious Doses) reduction of HTLV-III.[3]



Artist's interpretation of HTLV-III virus.

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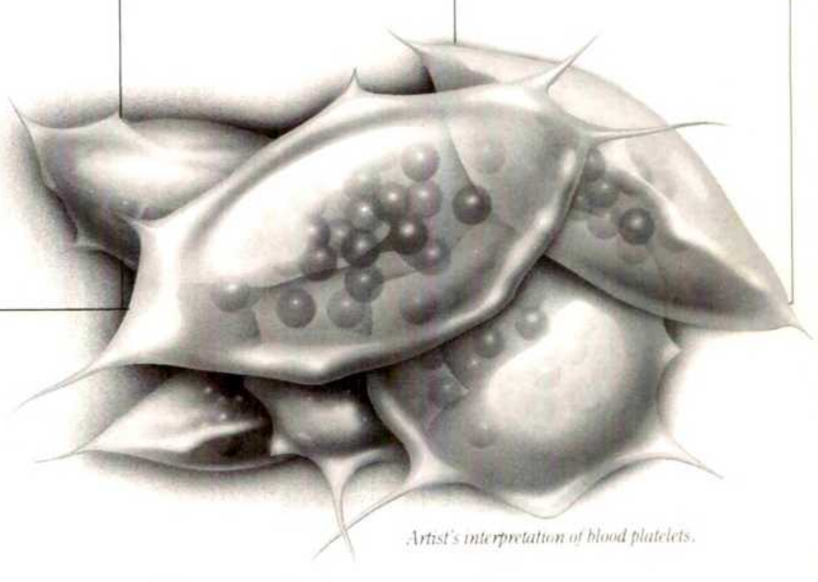

Artist's interpretation of blood platelets.

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[*] It is not possible to predict which patients with ITP will respond to therapy.

[†] In patients who respond to therapy, a rise in platelet count is generally rapid (within 1 to 5 days), transient (most often lasting from several days to several weeks) and should not be considered curative.

From the original IGIV, today's IGIV...it offers more

Please see following page for brief summary of prescribing information.

P25715

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: *Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.*

Idiopathic Thrombocytopenic Purpura (ITP): *Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.*

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: *Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.*

Drug Interactions: *If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.*

Pregnancy Category C: *Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.*

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: *The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.*

Idiopathic Thrombocytopenic Purpura: *An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.*

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be ***gradually*** *increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.*

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

U.S. License No. 8

REFERENCES: 1. *GAMIMUNE® N Product License Application.* **2.** *Lundblad JL, Mitra G, Steinberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. Rev Infect Dis 1986;8(Jul–Aug): S382–S390.* **3.** *Mitra G, Wong MF, Mozen MM, et al: Elimination of infectious retroviruses during preparation of immunoglobulins. Transfusion 1986;26(4):394–397.*

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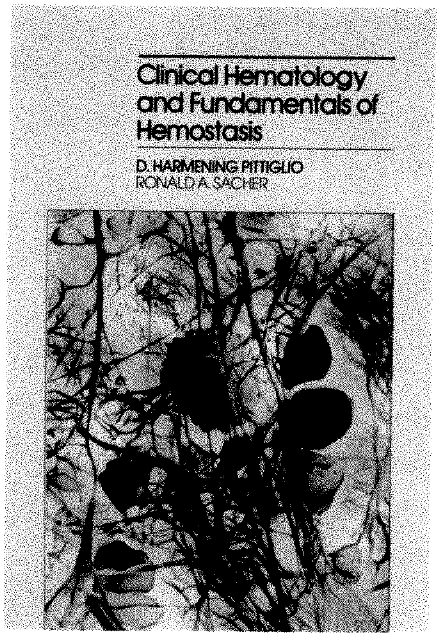

Clinical Hematology and Fundamentals of Hemostasis

D. HARMENING PITTIGLIO
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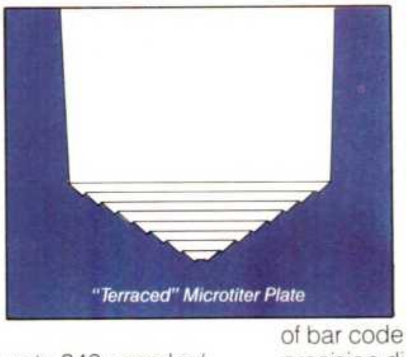
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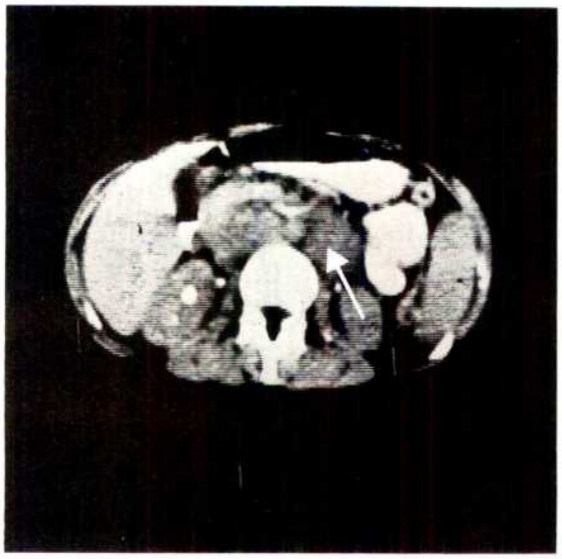
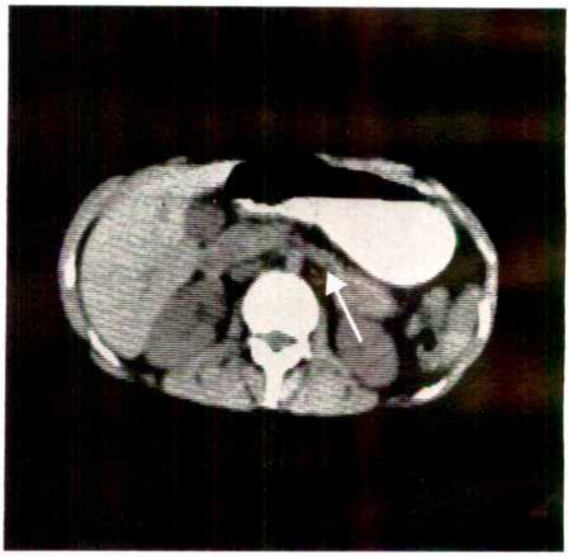


Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
August 4



No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al:* Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al:* Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

3. Laurence J, Coleman M, Allen SL, *et al:* Combination chemotherapy of advanced diffuse histiocytic lymphoma with the six-drug COP-BLAM regimen. *Ann Intern Med* 97:190-195, 1982.

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4. Skarin AT, Canellos GP, Rosenthal DS, *et al:* Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.
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m-BACOD

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WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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REVIEW

The Treatment of Hairy Cell Leukemia

By Harvey M. Golomb

IN VIEW OF THE INTRODUCTION of interferon for the treatment of hairy cell leukemia (HCL) in 1984[1] and its release by the Food and Drug Administration for this purpose in June 1986, it is important to consider its use in the context of all of the current indications and options for the treatment of patients with HCL. HCL has been thoroughly studied only for the past 12 years, even though it was first described in 1958 by Bouroncle et al.[2] The morphological, immunologic, and clinical characteristics of this disease have recently been reviewed.[3] It should be emphasized that, at diagnosis, the majority of patients have pancytopenia or multiple cytopenias, a result of the interplay between sequestration of blood in the spleen and underproduction of normal blood cells in the bone marrow. The need to effect a change in one or both sites determines the treatment modality chosen. It should be pointed out, however, that up to 10% of patients require no therapy at any time. Such patients are, on the average, 10 years older than the 52-year median for the whole group, and they have less splenomegaly, fewer circulating hairy cells, and more granulocytes than do other patients with HCL.[4]

TREATMENT

Treatment indications for HCL include severe anemia (hematocrit, <25%) as well as thrombocytopenia (platelet count, <50,000/μL), severe granulocytopenia (absolute granulocyte count, <500/μL), pain or other significant symptoms of splenomegaly, splenic rupture, repeated infections, bone involvement, vasculitis, and bulky retroperitoneal disease. Treatment options have included splenectomy, chemotherapy, leukapheresis, administration of androgens, bone marrow transplantation, radiation therapy, and biologic response modifiers (eg, interferon). There is no useful role for steroids except androgens in the treatment of HCL; when steroids have been given, no improvement in blood cell counts has occurred. Besides, steroids further compromise the immune system of an already abnormal host, thus increasing the risk of infection. Hairy cells have glucocorticoid-binding sites but are resistant to the cytolethal effects of glucocorticoids.[5,6] Another agent whose use should be discouraged is lithium. One patient was reported[7] to have improved hemoglobin levels and platelet counts with lithium therapy, but a subsequent study[8] and our own experience have not supported such a finding.

SPLENECTOMY

Splenectomy has been regarded as an attractive treatment option because it directly addresses the problem of splenic sequestration. However, we have learned that we cannot predict in individual cases whether patients will benefit from splenectomy because spleen size alone is not a direct indicator of postsplenectomy improvement in blood cell counts. In general, enlarged spleens are frequently associated with pancytopenia, and the counts of patients with significant splenomegaly respond more dramatically to splenectomy than is the case for smaller spleens.[9] Even in the absence of splenomegaly, however, significant cytopenia can occur, and it can respond to splenectomy.[10] Recently, we compared the presplenectomy and postsplenectomy blood cell counts of 170 previously untreated HCL patients.[3] The mean hemoglobin level increased from 10.4 g/dL to 11.5 g/dL ($P < .001$), the mean granulocyte count increased from 552/μL to 1754/μL ($P < .001$), and the mean platelet count increased from 87,000/μL to 211,000/μL ($P < .001$). Fewer than 7% of these patients had normal platelet counts before splenectomy; after splenectomy, this percentage rose to 63%. An analysis of the spleen weights of 153 of the 170 splenectomized patients showed that none were normal (<240 g); but of 24 nonpalpable spleens, six weighed between 241 and 400 g and 18 weighed between 401 and 600 g. Thus, approximately one sixth of the patients had nonpalpable spleens that could be two to three times the normal size. Although all HCL patients benefit from splenectomy, slightly less than half of the patients have improvement in all blood cell counts, and improvement in one or two components is seen in the remainder.[9] Moreover, we have updated our original observation that one third of patients require further therapy after splenectomy by showing that almost half (81 of

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166 patients) of the patients are in need of additional treatment.[3] The interval after splenectomy until further treatment is required is 1 to 164 months, with a median of 8.3 months.

CHEMOTHERAPY

Progressive disease following splenectomy occurs in two forms; half of the patients have pancytopenia caused by bone marrow replacement with hairy cells, and the other half have a leukemic form of the disease with an increasing white blood cell count (predominantly hairy cells). The latter group may have associated anemia, granulocytopenia, or thrombocytopenia.[11] For the leukemic form of progression, Yam and co-workers initially recommended leukapheresis.[12] Our experience, however, suggests that this does not result in long-term benefit.[13] Of four patients with the leukemic phase of the disease who underwent leukapheresis, two did not respond, and two had transient responses lasting for 1 to 6 months. We no longer utilize leukapheresis in the management of leukemic progression.

Progression with a leukopenic blood count results from underproduction of normal marrow elements secondary to infiltration of the bone marrow by hairy cells. Various types of chemotherapy as well as androgen therapy have been utilized for this form of the disease. In 1979, there were reports of improvement in blood cell counts with use of the alklyating agent chlorambucil in low doses[14] as well as with the drug rubidazone.[15] An update on the efficacy of chlorambucil in 24 HCL patients was published in 1984.[11] Patients were treated for at least 6 months with 4 mg chlorambucil given daily by mouth. Of 11 leukemic patients, all had a considerable dorp in their circulating hairy cells, and seven had increases in their platelet counts, in each case to more than 125,000/μL, following treatment. Of 13 pancytopenic patients, eight had substantially increased platelet counts after treatment; seven also had increased hematocrit values. Half of the patients with pretreatment and posttreatment bone marrow biopsies had objective improvement, as determined by a decrease in the number of hairy cells and an increase in normal marrow elements. However, there were six deaths, all of them associated with infectious complications.

In 1985, Calvo et al[16] reported on seven patients who were treated with intensive chemotherapy consisting of rubidazone, 450 mg/m^2, on day 1; cytosine arabinoside, 200 mg/m^2/d, from day 1 to day 5; and cyclophosphamide, 2,000 mg/m^2, on day 5. A complete response was defined as normal blood counts associated with the disappearance of hairy cell infiltration and fibrosis from the bone marrow. A partial response was defined as normal blood counts with persistence of leukemic cells in the bone marrow. Three patients achieved a complete response and one patient, a partial response. However, three patients died of infectious complications during induction chemotherapy. One patient, who had a complete response, died at 24 months while undergoing maintenance therapy. The remaining responding patients are alive at 44, 32, and 23 months. Joosten et al[17] reported on six HCL patients who relapsed after splenectomy and were treated with high-dose methotrexate (2 g/m^2) and leucovorin rescue. Five patients had objective responses, as determined by improved blood cell counts. The response continued for more than 14 months in one case and 44 months in another; however, these two patients had had the highest absolute neutrophil counts at the start of therapy.

In 1984, Spiers et al[18] reported on the treatment of two HCL patients with pentostatin (2-deoxycoformycin), a product of *Streptomyces antibioticus*. This agent is a tightly binding inhibitor of adenosine deaminase, an enzyme of purine metabolism. Hairy cells were cleared promptly from the blood, splenomegaly and lymphadenopathy regressed, and anemia, thrombocytopenia, and granulocytopenia were corrected. Both patients achieved a complete remission, which continued at 9 and 6 months, respectively, after their last treatment. Johnston et al[19] confirmed the activity of pentostatin in a single patient. At the same time, Spiers et al[20] reported on eight additional patients; three patients attained a complete remission and three were in partial remission (two remained on therapy and may achieve a complete remission). More recently, in 1986, Johnston et al[21] reported eight patients treated with pentostatin. Complete remission occurred in two patients, and four patients had 50% to 95% marrow clearance of hairy cells. Kraut et al[22] reported 10 patients with progressive hairy cell leukemia treated with low-dose pentostatin (4 mg/m^2 IV every other week); nine had a complete remission. Seven patients remained in unmaintained remission with a median duration of 6.2 months. A major advantage of pentostatin is that complete bone marrow remission can be achieved, and may be durable.

HORMONAL THERAPY

Androgens have been used for the treatment of HCL with some success. One study showed that three of six patients who did not benefit from splenectomy responded to androgen therapy, with correction of cytopenia.[23] The responses occurred 1 to 20 weeks after the initiation of therapy and lasted for at least 17 months. A second study[24] concerned a single patient who required an RBC and platelet transfusion after splenectomy. Within 2 months after the beginning of oxymetholone therapy (50 mg orally three times a day), the patient's platelet count had normalized, and improvement in erythocyte and leukocyte counts followed. When the drug therapy was discontinued, the peripheral blood cell counts deteriorated drastically, but they improved when oxymetholone treatment was reinstated. We had a patient who progressed after splenectomy. He did not respond to chlorambucil and continued to require RBC transfusions. Treatment with oxymetholone at the aforementioned dosage led to a normalization of all blood cell counts. The patient has not been receiving oxymetholone for several years and has maintained normal counts.

RADIATION THERAPY

The role of radiotherapy in HCL is limited to the treatment of local problems. Bone involvement has been shown to occur in HCL patients.[25] We have reported six patients who developed hip pain 9 to 73 months (median, 23 months) after the initial diagnosis.[26] As seen on roentgenographs, five patients had single or multiple osteolytic lesions in the proximal portion of the femur, and one patient had no abnormalities. One patient had a concomitant subcapital femoral fracture, and another later developed aseptic necrosis of the proximal parts of the femur and humerus. Five patients were treated with irradiation in doses ranging from 1,500 to 3,000 rad. Three patients completely improved, but two had persistent mild hip discomfort. Orringer and Varia[27] described a patient who developed massive retroperitoneal, abdominal, and mediastinal lymphadenopathy 2 years after splenectomy. Low-dose radiation (600 to 750 rad), given for disabling pain in the back and chest, produced prompt regression of the adenopathy as well as pain relief. The response of patients with these types of local problems suggests that HCL is a radiosensitive disease and that local treatment should be used for local problems.

INTERFERON

The use of α-interferon in the treatment of HCL was first recommended in 1984 by Quesada et al[28] who reported seven patients treated with partially purified leukocyte interferon-α. Granulocytopenia and thrombocytopenia were reversed within several months of therapy. There were three complete responses, and six patients had less than 5% hairy cells in their bone marrows within 8 to 12 weeks.

Because we were following a large group of HCL patients (currently, more than 300 patients), we were able to institute a trial of recombinant α_{2b}-interferon (2×10^6 U/m^2 subcutaneously [SC] three times per week) in October 1983. In eight evaluable patients, we also obtained dramatic results, with seven partial responses,[29] which confirmed that interferon was the active agent. Foon et al[30] recently published their results for 15 patients treated with α_{2a}-interferon, which differs from the α_{2b} form only in the amino acid at position 23. Patients were treated with 3×10^6 units daily either intramuscularly (for platelets $>50{,}000/\mu L$) or SC (for platelets $<50{,}000/\mu L$). They noted one complete response and 12 partial responses for a response rate of 87%. Coincident with the normalization of peripheral blood counts, natural killer cell activity returned, and immunologic surface markers became normal. Comparable results have been obtained with a highly purified polyclonal interferon-α preparation from lymphoblastoid cells.[31]

Quesada et al[32,33] recently summarized their experience in treating 52 HCL patients with interferon; 22 had received leukocyte interferon-α and 30, α_{2a}-interferon. There were five complete, 13 partial, and four minor responses among the 22 patients and five complete, 17 partial, and six minor responses among the 30 patients. The total complete and partial response rate was thus 77%, and the overall response rate was 96%. Six patients had splenomegaly and were candidates for splenectomy, but they elected to have α-interferon as primary therapy. In all six patients, the size of the spleen decreased rapidly (within seven to 15 days). Five of the six obtained a complete remission, compared with four of 24 patients on whom a splenectomy had been performed prior to interferon therapy. Thus, the efficacy of α-interferon in previously untreated patients was demonstrated.

A multicenter study of 64 HCL patients treated with α_{2b}-interferon was recently published by Golomb et al.[34] Three patients (5%) had a complete response, with apparent eradiction of hairy cells from the bone marrow; 45 patients (70%) had a partial response, defined as normalization of all three blood cell counts and decreased HCL involvement in the bone marrow; and nine patients (14%) had a minor response that included improvement in a least one blood cell count. Thus, the overall response rate was 89%. Three patients had no response, three patients died before completing 1 month of therapy, and one patient refused further therapy after being treated for 1 month. A rapid return of a normal platelet count (within 2 months) was confirmed; the hemoglobin and granulocyte counts returned more slowly (within 4 and 5 months, respectively). Subsequent bone marrow biopsy analyses showed a decrease in the hairy cell index by more than half. Serious infections, which had averaged four per month in 16 of the 64 patients before interferon therapy, occurred only rarely after the first month of treatment. Treatment-induced toxic effects were mild and consisted of influenzalike symptoms, fatigue, and minor skin disorders.

The same group has prepared an updated evaluation of the first 128 HCL patients treated with α_{2b}-interferon.[35] The responses obtained were almost identical to those for the first 64 patients. There were five complete responders (4%), 98 partial responders (77%), and nine minor responders (7%). The overall response rate is thus at 88% for the 128 patients. In contrast to Quesada's[32] group of previously untreated patients who had significant complete responses, the patient group[35] includes 13 patients who had not undergone splenectomy; but none of these were complete responders. The discrepancy between the two reports remains to be explained.

Thus, the percentage rate of response to recombinant α-interferons is very high in a large number of patients. This finding led to approval, in June 1986, of recombinant α-interferon by the FDA for prescription for HCL patients by practicing physicians. It is important that the exact role of α-interferon in HCL be understood.

Two groups of investigators recently addressed the objective changes in the peripheral blood and bone marrow of HCL patients who were treated with recombinant α-interferon. Naeim and Jacobs[36] studied blood and bone marrow specimens from 21 patients. Bone marrow samples at 3 months after initiation of interferon therapy showed a decrease in cellularity from 77% to 57%, with a marked decrease in the percentage of hairy cells (87% to 59%). Increased amounts of reticulin fibers persisted after completion of interferon therapy, as did a high incidence of dry taps. They found that with interferon therapy the tartrate-resistant acid phosphatase (TRAP)-positive HCL cells became TRAP-negative, thus suggesting inhibition of activity and/or production of TRAP in HCL cells. Bardawil et al[37] studied specimens from 12 patients. When they examined the patients after 6 to 12 months of interferon therapy, they found that the median hairy cell index had decreased from 0.56 to 0.12; but in no case were hairy cells completely absent from the bone marrow. The numbers of large granular lymphocytes increased in 11 of the 12 patients after interferon therapy, but the numbers often fluctuated, and in three patients they later fell to preinterferon values.

It is important to begin an assessment of the durability of response in patients after interferon therapy is discontinued. Two groups of investigators are attempting to do this. Quesada et al[33] observed a clinical relapse in eight of 25 patients (33%) in whom treatment was discontinued after 12 months. The clinical relapse occurred within 3 to 10 months (median, 6 months) after cessation of treatment in six patients who attained partial remission and one patient each with a complete remission and minor response. Ratain et al[38] have studied 24 patients who completed interferon therapy a median of 8.5 months (range, 3 to 16 months) previously. Only one patient has required further treatment. However, in general there was a marked decrease in normal marrow elements and a relative increase in peripheral blood counts. Clearly, more patients need to be observed closely over a longer period.

In our multicenter study,[35] we attempted to address the issue of duration of therapy and durability of response. A group of patients completing 12 months of interferon therapy and having a minor, partial, or complete remission was randomized to an additional 6 months of interferon therapy or to observation alone. A very preliminary analysis reveals six of 35 patients (17%) randomized to observation alone have relapsed, whereas only two of 29 (7%) in whom interferon therapy was continued have relapsed. Twenty-seven responding patients remained off interferon treatment for 6 months or more. Thus, it has been demonstrated that cessation of interferon therapy does not result in a rapid deterioration of peripheral blood counts. The number of relapsing patients is still too small, however, to allow significant conclusions.

A few HCL patients have not responded to α-interferon. The question arises whether they can respond to pentostatin. Foon et al[39] recently reported two patients with massive splenomegaly and severe pancytopenia who had no significant response in a trial of α_{2a}-interferon. Subsequent treatment with pentostatin for 8 consecutive weeks resulted in normalization of spleen size, peripheral blood cell counts, and bone marrow in one patient. The second patient had a reduction in spleen size and improved blood cell counts following 9 weeks of pentostatin therapy. We have recently observed a patient whose disease progressed during his 14th month of α_{2b}-interferon therapy. He did not respond to treatment for 2 months with γ-interferon and remained dependent on platelet transfusions. Six courses of pentostatin (4 mg/m^2 intravenously every 2 weeks) therapy led to eradication of the hairy cells in the bone marrow and a return to normal peripheral blood cell counts. It thus appears that pentostatin can be effective in patients whose disease has progressed during α-interferon therapy.

RECOMMENDATIONS

What can we conclude from the published and about-to-be-published information with regard to the treatment of patients with HCL? In my opinion, after the correct diagnosis of HCL has been made, one must attempt to identify the 10% of elderly patients who have adequate blood counts and a nonpalpable spleen and who require no treatment. The remaining 90% of patients will usually require some form of therapy within a short period after the diagnosis. For a patient with an easily palpable spleen, low blood counts, and a bone marrow that shows only patchy involvement, a splenectomy should be undertaken as the first choice of treatment. The probability is high that the peripheral blood counts will improve and that the patient might require no further therapy for many years. For a patient with a nonpalpable spleen, low blood cell counts, and a diffusely involved bone marrow, systemic therapy is the first choice. Such patients probably gain little benefit from splenectomy, and they will require systemic therapy within a short time after splenectomy. For a patient who fits between these two categories, either option is reasonable, and both options should be considered and discussed with the patient.

The type of systemic therapy chosen for the presplenectomy patient who will benefit from it or for the postsplenectomy patient who has progressive disease and requires it depends on the following considerations. Interferon is highly active in HCL, with a high response rate, but it results in a low percentage of complete remissions. However, it controls the disease very well and leads to reversal of the clinical problems. Cessation of interferon therapy does not bring about a rapid deterioration of peripheral blood cell counts. Furthermore, a response can be reinduced in the majority of patients requiring retreatment, and the toxicity is mild by comparison with most forms of standard chemotherapy. Pentostatin has been tested in fewer patients; however, it yields a much higher complete response rate and requires only 3 months of therapy as compared with 12 months recommended for interferon. The duration of complete remission is excellent, but the toxicity in terms of the immediate effect on renal and liver function and the delayed effect on immune function remains to be determined.

A randomized trial of pentostatin and α-interferon in postsplenectomy patients with progressive disease is currently under way by the Cancer and Leukemia Group B (CALGB). From the results it should be possible to draw conclusions about the aforementioned concerns. It should also be restated that α-interferon is now available clinically (as of June 1986), whereas pentostatin is not (and is tightly controlled by the National Cancer Institute). However, pentostatin is available through a national cooperative group (CALGB) for HCL patients who have failed α-interferon therapy.

Thus, for patients not being entered into clinical trials, I recommend that α-interferon be used as the first systemic treatment of choice until more data on pentostatin become available regarding its efficacy and toxicity in HCL patients. Also, because pentostatin is effective in patients who have not responded to α-interferon, its benefits can still be utilized by patients initially treated with interferon. Thus, in the event that pentostatin becomes the first agent of choice for systemic treatment of HCL in the future, current patients with progressive disease will have received its therapeutic benefits.

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Chromosome Aberrations and Clinical Features of Adult T Cell Leukemia-Lymphoma Not Associated With Human T Cell Leukemia Virus Type I

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Chromosome aberrations and clinical features of three patients with adult T cell leukemia-lymphoma (ATL) not associated with human T cell leukemia virus type I (HTLV-I) are described. From their clinical features, two patients were diagnosed as acute type and one patient was diagnosed as chronic type, which later converted to acute crisis. Clonal and many chromosomal abnormalities were observed before therapy in the two acute type cases and at relapse in the chronic type case. Karyotype aberrations, including trisomy 3, trisomy 7, trisomy 21, del(6)(q21), del(10)(p13), 14q11 translocation, and loss of X chromosome, all of which are frequently found in HTLV-I associated ATL, were also seen in these cases of HTLV-I–negative ATL.

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WE SHOWED in 1983 that some patients with ATL have typical clinicohematologic, morphological, and immunophenotypic features, but test negative for antibody to human T cell leukemia virus type I (HTLV-1).[1] By Southern blot analysis, we recently proved that HTLV-1 was not associated in five patients with anti–HTLV-I antibody-negative ATL.[2] These findings suggested that HTLV-1 may not be involved in leukemogenesis of some ATL patients. To examine leukemogenesis and the exacerbation process of such ATL patients, we conducted karyotype analysis of the leukemia cells. We present evidence that chromosome abnormalities similar to those found in typical HTLV-1-associated ATL were also found in HTLV-1–negative ATL.

MATERIALS AND METHODS

Patients. Previously,[2] we confirmed that any nucleotide sequences of the provirus genome of HTLV-I were not integrated into DNA of leukemia cells from all five patients with anti–HTLV-I antibody-negative ATL. In these patients, the leukemia cells had flower cell morphology as well as the inducer/helper phenotype, both of which are characteristics of ATL.[3-6] Karyotype analysis could be made in the leukemia cells from three of the five patients with HTLV-I–negative ATL. The patients were classified according to clinical subtypes of ATL, as described elsewhere.[4-7]

Cytogenetics. Cytogenetic studies were done on specimens of peripheral blood and pericardial effusion which were obtained before therapy and at relapse. Mononuclear cells prepared from these sources by the usual Ficoll-Hypaque gradient method were examined according to conventional techniques[8] after culturing for 18 to 48 hours without mitogens such as phytohemagglutinin or T cell growth factor. After hypotonic treatment with KCl (0.075 mol/L) for 20 minutes, the cells were fixed in methanol acetic acid (3:1), dropped onto glass slides, and air dried. Conventional Q-banding and/or Giemsa techniques were applied to the slides. Metaphase and prometaphase cells were photographed and then arranged according to the International System for Human Cytogenetic Nomenclature (ISCN).[9] The criteria adopted at the First International Workshop on Chromosomes in Leukemia[10] were used to identify abnormal clones. Karyotypes of cases 1 and 2 were determined after complete reviews by expert members of ATL Karyotype Review Committee—1985.

RESULTS

Clinical features. The clinical features of the three patients with HTLV-1–negative ATL are summarized in Table 1. The age at onset of the disease ranged from 43 to 71 years. There were two men and one woman. Two patients (cases 1 and 2) became acutely ill, and their symptoms at onset were subfever, lymph node swelling, skin rash, and hepatosplenomegaly. Leukemic manifestation and high serum LDH levels were also observed. Pulmonary infiltration of leukemia cells developed later. The patients died of the disease 3 and 11 months, respectively, after the diagnosis (Table 1). In another patient (case 3), leukocytosis due to abnormal inducer/helper T lymphocytosis of flower cell morphology was accidentally found when the patient complained to his physician of dizziness due to iron-deficiency anemia. His T lymphocytosis remained unchanged for ~2 years without specific therapy and then converted to acute phase. Skin lesions, lymph node swelling, hepatosplenomegaly, pulmonary infiltration, pleural effusion, and pericardial effusion developed. Hypercalcemia developed later, and the patient died of cardiac insufficiency 34 months after the diagnosis and 3 months after the acute transformation (Table 1).

Hypercalcemia was observed in two patients. Immunoglobulin levels were found to be within the normal range at onset of the disease in all three patients. Combination chemotherapies, including vincristine, cyclophosphamide, prednisolone, and doxorubicin (VEPA[11] or CHOP[12] therapy), were effective. Two patients were induced into remission, but the effect was transient. From the clinical features,

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Table 1. Clinical Features

Factor	Case No. 1	Case No. 2	Case No. 3
Age at onset, sex	71, M	43, F	47, M
Complaints at presentation	LN swelling, anorexia, subfever, hoarseness, cough	LN swelling, erythrodermia, subfever	Dizziness, back pain
Organ involved			
Skin	−	+	− → +
Lung	− → +	− → +	− → +
Lymph nodes	+	+	− → +
Liver	+	+	− → +
Spleen	+	+	− → +
Pleural effusion	− → +	−	− → +*
Laboratory examination			
WBC (per μL)	56,800	74,600	17,300
Abnormal L	64%	87%	72%
Abnormal L in BM	54%	36%	11%
LDH (IU/dL)	2,610	1,163	370 → 1,717
Hypercalcemia	− → +	−	− → +
Immunoglobulin	N	N	N
Treatment and response	VEPA NR	CHOP, MTX CR	CHOP, MTX PR
Survival after diagnosis (mo)	3	11	34
Diagnosis	ATL, acute type	ATL, acute type	ATL, chronic type

LN, lymph node; WBC, white blood cell; L, lymphocyte; BM, bone marrow; LDH, lactic acid dehydrogenase; N, normal; NR, no remission; PR, partial remission; CR, complete remission; ATL, adult T cell leukemia-lymphoma.

*Pericardial effusion developed later.

cases 1 and 2 were diagnosed as acute type and case 3 was diagnosed as chronic type, according to the classification of subtypes of ATL[4-7] (Table 1).

Cytogenetic findings. Karyotype analysis was made before therapy in all three patients and again at relapse in two (cases 2 and 3). The results are summarized in Table 2. In one patient (case 1), chromosome constitution was extremely complicated: Among abnormalities, +der(2)t(2;?)(?;p16q31;?), -6, 6p-, and breaks at the proximal portion of chromosome 14 [14p11 and 14q11→+der(14)t(14;?)(p11;?) and +der(1)t(1;14)(p11;q11)] were noted (Fig 1). In case 2, partial trisomy 3 and

Table 2. Cytogenetic Findings

Case No.	Source of Cells	Modal Chromosome Constitution	No. of Cells Observed
1	24-h PB	a: 43,X, −1, −2, −6, −8, −9, −9, −10, −13, −14, −14, −15, −16, −18, −20, +21, −22,t(Y;4)(q11;q31), +der(1)t(1;14)(p11;q11), +der(2)t(2;?)(?;p16q31:?),del(6)(p21), +der(9)t(9;?)(p22;?), +der(13)t(13;?)(p11;?) + der(14)t(14;?)(p11;?), +der(15)t(15;?)(p11;?), +der(18)t(18;?)(p11;?), +mar1, +mar2, +mar3, +mar4.	46/69
		b: 4n+ of a.	23/69
2	24-h and 48-h PB	c: 48,X, −X, −5, −12, +der(3)t(3;?)(q12;?), +der(5)t(5;12)(q22;q14), +mar1, +mar2, +min.	10/10
	24-h PB*	d: 47, −X, −5,t(X;6)(q12;p22), +der(3)(3;?)(q12;?), +der(5)t(5;12)(q22;q14),del(12)(q14), +min.	19/27
3	24-h PB	e: 46,XY.	18/18
	18-h PE*	f: 46,XY, −4, −11, −12, −17, −21,del(3)(p12), +der(4)t(4;?)(q31;?),del(6)(q21q23),del(8)(p21),del(10)(p13), +der(11)t(11;?) −(p15;?), +der(12)t(12;?)(p13;?), +der(21)t(17;21)(q11;p13), +der(21)t(17;21)(q11;p13).	3/10
		g: 45,X, −Y, −11, −12, −17, −21, −21,del(3)(p12),del(6)(q21q23), +der(7)t(7;?)(q22;?),del(10)(p13), +del(11)t(11;?)(p15;?), +der(12)t(4;12)(q11;p13), +der(21)t(17;21) −(q11;p13), +der(21)t(21;?)(p13; ?).	2/10

PB, peripheral blood; PE, pericardial effusion.

*Cytogenetic study was performed at relapse.

t(5;12)(q22;q14) were found in addition to three marker chromosomes (Fig 2A). Chromosome constitution obtained at relapse showed almost the same as that obtained before therapy. But, a translocation involving an X chromosome-t(X;6)(q12;p22)-was newly found, suggesting clonal evolution (Fig 2B). On the other hand, in case 3, the karyotype remained apparently normal, and no gross abnormality was found before therapy. At relapse, however, cells in the pericardial effusion also showed very complicated karyotypes (Fig 3A and B). Two abnormal clones were determined, one of which had a missing Y and partial trisomy 7 (Fig 3B). Many rearranged chromosomes, including del(3)(p12), del(6)(q21q23), del(10)(p13), +der(11)t(11;?)(p15;?), +der(21)t(17;21)(q11;p13) were common to each other, however, suggesting that both cell lines originated from the same progenitor cell. Thus, all three patients had complicated chromosome constitutions, although their modal numbers were near-diploid range. Abnormalities common to three patients were not detected. Abnormalities similar to those found in HTLV-I–associated ATL[13-25] were also found in these three cases of HTLV-I–negative ATL, however.

DISCUSSION

We present the chromosome aberrations and clinical features of three patients with ATL not associated with HTLV-1. These findings and the hematologic, pathologic, and immunophenotypic features of these cases as reported elsewhere[2] are identical to those of HTLV-1–associated ATL.[4-7] The patients in this report are the same as the patients in the previous report,[2] in which we proved that the patients showed neither integration of the HTLV-I proviral genome into chromosomal DNA of leukemia cells nor anti–HTLV-I antibody in serum. In addition, no anti–HTLV-I antibody was detectable in any family members. These facts indicate that HTLV-I was not associated with leukemogenesis in any of these cases of ATL, but we could not exclude the possible presence of a small amount of anti–HTLV-I antibody and of a very small fragment of the HTLV-I provirus that could not be detected with current techniques. Diversity in the clinical course of HTLV-I–associated ATL has been reported.[4-7] Like HTLV-1–associated ATL, there was also diversity in the clinical course of HTLV-1–negative ATL, ie, acute type and chronic type were observed in HTLV-1–negative ATL in this series.

Clonal and many chromosomal abnormalities are documented for HTLV-I–associated ATL by karyotype analysis.[13-19,24,25] Rowley and colleagues[25] reported that trisomy for chromosomes 3 and 7 or 7q, and monosomy for X chromosome, 14q+, and 6q− were noted as the most common abnormalities in ATL, but 14q+ abnormality was not identified in any of the Caribbean ATL patients. Recently, karyotype analysis by the ATL Karyotype Review Committee—1985 of 78 ATL patients from 13 institutions in Japan revealed that +3, +7, -X rearrangements involving 6q15, 6q21, 10p11–13, 14q11, and 14q32 were the most common abnormalities in ATL.[26] In the early phases of ATL, however, such as in smoldering ATL,[4-7] or in chronic ATL,[5,7] minimal chromosomal abnormalities have been reported.[18,20]

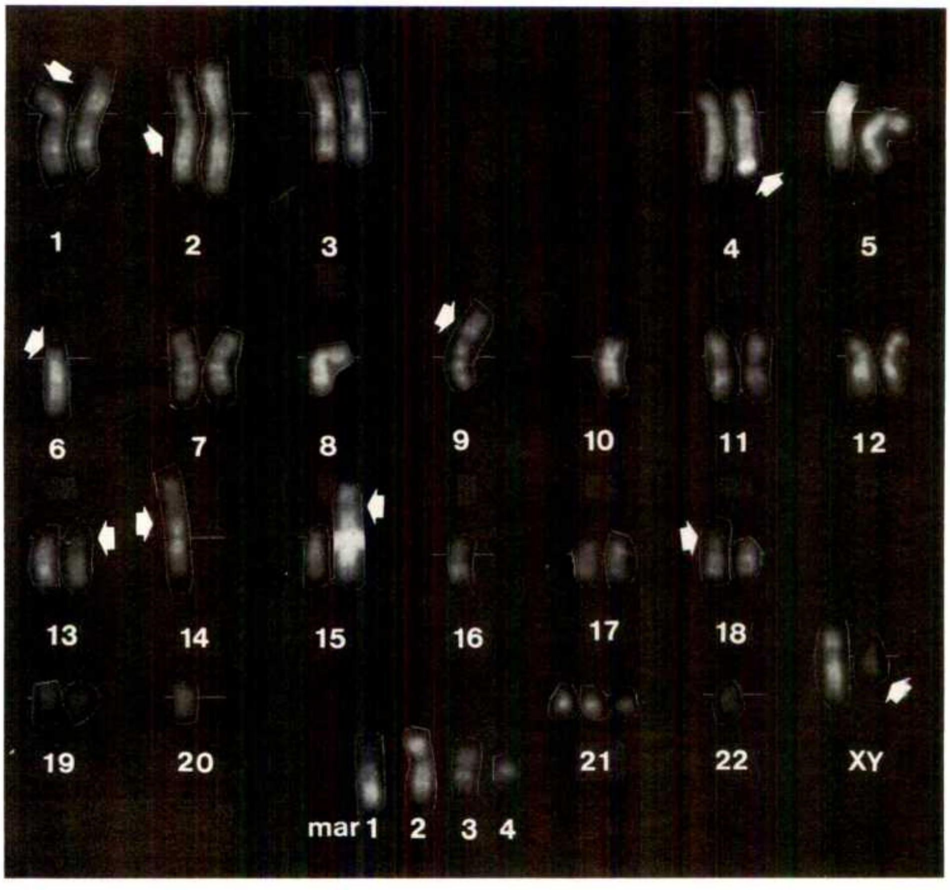

Fig 1. Q-banded karyotype of a leukemia cell from case 1. Exact karyotype is shown in Table 2(a).

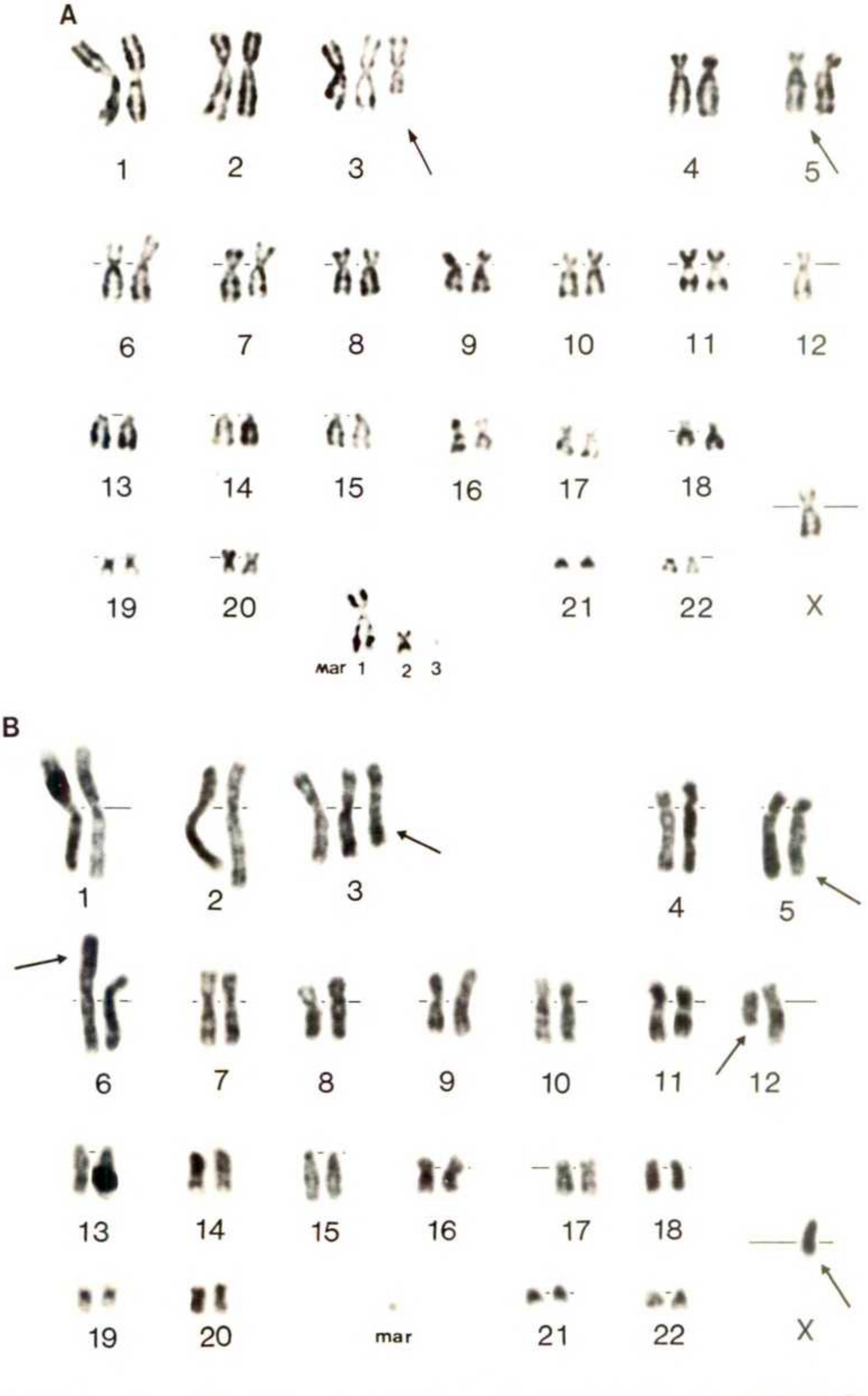

Fig 2. G-banded karyotype of a leukemia cell from case 2. (A) Before therapy. Exact karyotype is shown in Table 2(c). (B) At relapse. Exact karyotype is shown in Table 2(d).

As presented here, in acute HTLV-1–negative ATL, clonal and many chromosomal abnormalities were observed. In chronic HTLV-1–negative ATL (case 3), no chromosomal abnormalities were found at chronic phase, but clonal and many abnormalities, including del(6)(q21q23), +der(7)t(7;?)(q22;?), and del(10)(p13), were found at relapse. In case 1, abnormalities involving the proximal portion of chromosome 14, +der(1)t(1;14)(q13;q11) were found. Recently, abnormalities involving 14q11-q13 have been increasingly reported in various T cell malignancies, including ATL.[16,19,21-23,26,27] In case 2, trisomy for chromosome 3, +der(3)t(3;?)(q12;?), and monosomy for X chromosome were observed. From these cytogenetic findings, it is speculated that HTLV-1–negative ATL cells also have chromo-

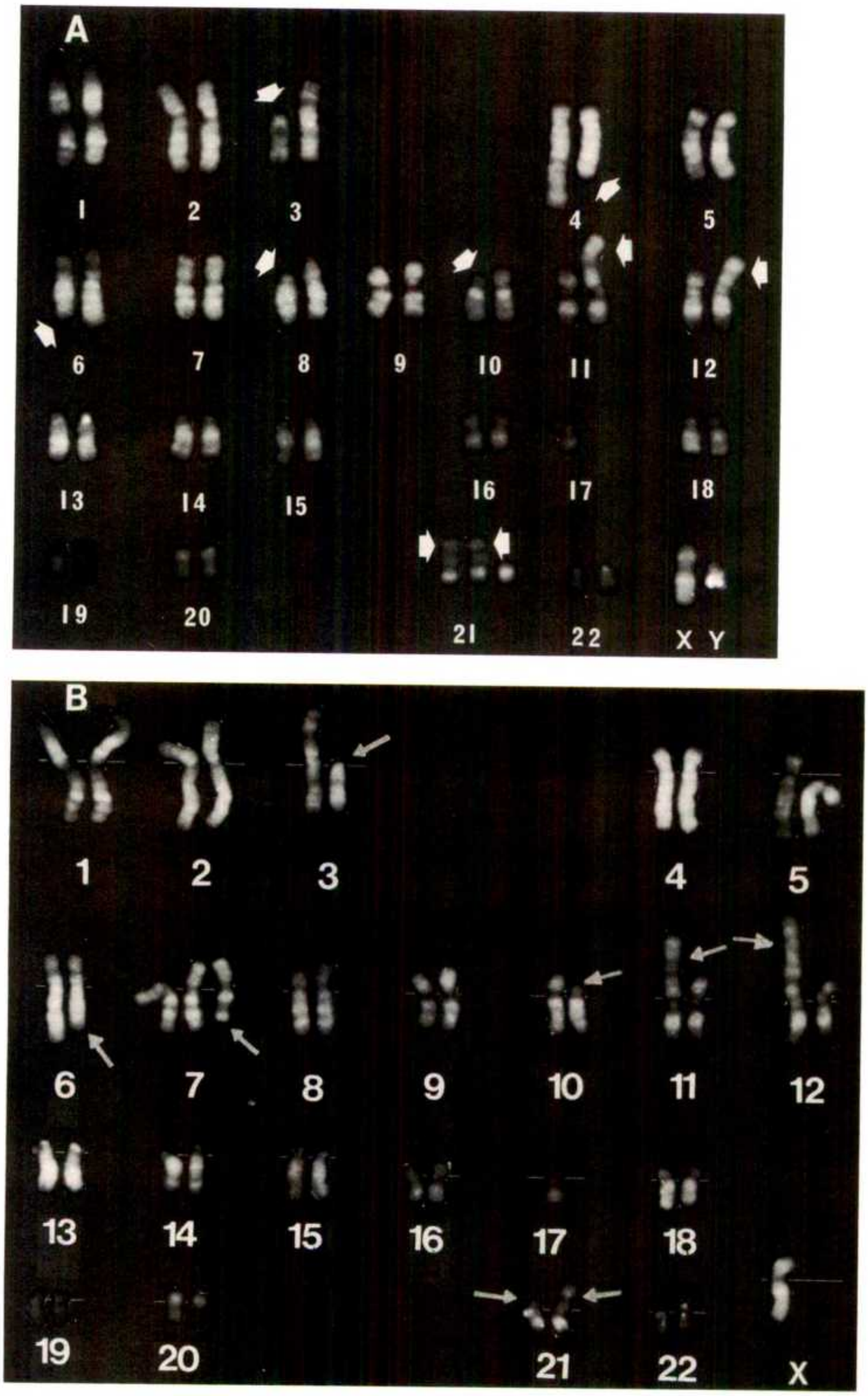

Fig 3. Q-banded karyotype of a tumor cell in pericardial effusion of case 3. Two clones, A and B, were observed. Exact karyotypes of clones A and B are shown in Table 2(f) and 2(g), respectively.

somal abnormalities similar to those reported in HTLV-1–positive ATL or other T cell malignancies.

The number of HTLV-I–negative ATL cases reported is very small, indicating a need for close scrutiny of this disease. Results indicate no detectable differences between the clinicohematologic, cytopathologic, immunologic, and karyotype features of HTLV-1–positive and HTLV-1–negative ATLs. The findings of HTLV-1–negative ATL suggest that there is a similar underlying mechanism(s) in leukemogenesis as well as in the malignant process of both HTLV-I–related and HTLV-I–unrelated ATL. The presence of HTLV-1–negative ATL suggests the existence of unknown cellular oncogenes closely related to the transformation of inducer/helper T cell. We consider the role of HTLV-I in the etiology of ATL to be similar to that of Epstein-Barr virus in Burkitt's lymphoma. The causative agent of ATL may not always be

HTLV-I; hence, the disease entity of ATL may not always be defined as an HTLV-I–induced T cell malignancy.

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Establishment and Characterization of a Cell Line, TOM-1, Derived From a Patient With Philadelphia Chromosome–Positive Acute Lymphocytic Leukemia

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A new Philadelphia chromosome (Ph^1)-positive cell line, designated TOM-1, was derived from bone marrow cells of a patient with Ph^1-positive acute lymphocytic leukemia (ALL). The TOM-1 cells were positive for Ia and B1 antigens and terminal deoxynucleotidyl transferase (TdT) but negative for common ALL antigen. Although neither surface Ig nor cytoplasmic Ig was detected, the TOM-1 cells contained rearranged immunoglobulin-H chain genes but retained germ-line κ chain and germ-line T cell receptor β-chain genes. These results indicate that the TOM-1 cells reside as the progenitor of pre-B cells. We have investigated the chromosome 22 breakpoint and c-*abl* gene expression in the TOM-1 cells. We found that the breakpoint on chromosome 22 was within the breakpoint cluster region (*bcr*) in the TOM-1 cells. We also found the breakpoints within or near *bcr* in four of six Ph^1-positive ALL cases, similar to the findings in Ph^1-positive CML cases. Amplification of the c-*abl* gene was not detected in the TOM-1 cells. The leukemic cells isolated from a patient with CML in myeloid crisis contained a novel 8-kilobase (kb) *abl*-related messenger RNA (mRNA), but the TOM-1 cells contained c-*abl* transcripts of only normal sizes, despite the fact that they showed the *bcr* gene rearrangement.

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THE PHILADELPHIA (Ph^1) chromosome is a typical cytogenetic abnormality observed in chronic myelogenous leukemias (CML).[1] However, the Ph^1 chromosome has also been observed frequently in acute lymphocytic leukemias (ALL),[2,3] since Propp and Lizzi reported a case of Ph^1-positive ALL.[4]

It has been generally accepted that the development of cell lines derived from various types of hematopoietic disorders may offer the means by which to resolve the pathophysiology of the diseases. Several Ph^1-positive leukemia cell lines have been established from patients with CML in blastic crisis[5-7] and have offered opportunities for analysis of the molecular events accompanied by the t(9:22) translocation.[8,9]

It has been reported that, in CML cells, the c-*abl* gene translocated from chromosome 9 to chromosome 22 and aberrant 8-kb c-*abl* mRNA, longer than that of normal counterparts, was transcribed.[9-11] In CML the breakpoints on the chromosome 22 cluster are within approximately a 5.8-kb segment, which is referred to as breakpoint cluster region (*bcr*).[10] These findings strongly suggest the role of c-*abl* and *bcr* genes in the pathogenesis of CML. At present it is unknown whether or not the breakpoint on chromosome 22 and the abnormal expression of the c-*abl* gene found in the CML cells can be extended to Ph^1-positive ALL.

We have established a new leukemic cell line, designated as TOM-1, from a patient with Ph^1-positive ALL. This cell line, as far as we are aware, is the first cell line derived from a Ph^1-positive ALL patient. This cell line was identified as the progenitor of pre-B cells, as revealed by analyses of cell surface antigens and Ig gene rearrangements. We report here *bcr* gene rearrangement and expression of c-*abl* gene in the TOM-1 cells.

CASE REPORT

A 54-year-old female was referred to the Department of Internal Medicine, Kanazawa Medical College, on September 9, 1983, displaying marked leukocytosis (WBC count: 42.5 × 10^9/L). The diagnosis was ALL, which phenotypically was Ia-positive and common ALL antigen (CALLA)-positive. She then received combination chemotherapy with vincristine, cyclophosphamide, Adriamycin (doxorubicin, Farmitalia, Milan, Italy) and Predonin (prednisolone, Schering, Kenilworth, NJ); but remission was not achieved. She was referred to our hospital on November 14, 1983. On admission, her peripheral blood examination revealed RBC count 415 × 10^{10}/L and WBC count 8.2 × 10^9/L with no appearance of leukemic cells. The bone marrow aspirate showed hypercellular marrow (nuclear cell count: 85.9 × 10^{10}/L) consisting of 89.7% leukemic cells. The Ph^1 chromosome was identified in all bone marrow cells analyzed. In May 1984, complete remission was achieved after MOAD therapy: moderate-dose methotrexate, vincristine, asparaginase, and dexamethasone.[12] At that time the Ph^1 chromosome was not detected in bone marrow or peripheral blood cells. After remission was achieved, a transversion into chronic CML was not found. However, the patient relapsed and died of respiratory failure due to bacterial pneumonia in July 1984.

MATERIALS AND METHODS

Culture of leukemic cells. Heparinized blood was taken by bone marrow aspiration from the patient on November 14, 1983. Mononuclear cells were separated by Ficoll-Hypaque density sedimentation, washed twice, and suspended in complete medium; RPMI 1640 was added with 10% fetal bovine serum, 2 mmol/L L-glutamine, 25 mmol/L Hepes buffer, 100 U/mL penicillin G, and 100 μg/mL streptomycin. The leukemic cells were cultured in tissue culture plastic flasks in humidified atmosphere added with 5% CO_2 at 37 °C.

Cytochemistry. Staining with periodic-acid Shiff (PAS), peroxidase, acid phosphatase, Sudan Black B (SBB), α-naphthyl butylate esterase (α-NBE), and naphthol AS-D chloroacetate esterase (NACE) was performed on primary and established leukemic cells.

Phenotypical analysis. Surface immunoglobulin (sIg) and cytoplasmic immunoglobulin (cIg) were analyzed by direct immuno-

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Table 1. Clinical Profiles of the Patients with Ph^1-Positive ALL

Case	Age/Sex	FAB Classification	Phenotypical Markers						Karyotype Analysis		
			OKIa1	J-5	Pan-B	OKT3	OKM1	TdT	Karyotype	Prior to Tx	Post Tx
1	59/F	L2	100	70	40*	<1	NT	NT	46,XX,t(9q+:22q−)	95%	0%†
									46,XX	5%	100%
2	35/M	L2	97	80	3*	4	3	+	46,XY,t(9q+:22q−)	75%	77%§
									46,XY	25%	23%
3	58/M	L2	95	53	44*	1.8	4.8	+	46,XY,t(9q+:22q−)	100%	67%§
									46,XY	0%	33%
4	41/M	L1	80	90	94‖	2.1	10	+	49,XY,+6q−,t(9q+:22q−)	100%	0%†
									46,XY	0%	100%
5	37/F	L2	77	69	73‖	3.4	4.2	+	46,XX,+22q−,t(9q+:22q−)	43%	58%‡
									46,XX	57%	42%
6	46/F	L2	85	80	70‖	1.0	9.6	NT	45,XX,−7,t(9q+:22q−)	100%	0%†
									46,XX	0%	100%

Abbreviations: Tx, therapy; NT, not tested.

*OKB1.

†In complete remission.

‡In no response.

§In partial remission.

‖OKB2.

fluorescence methods. Fluorescein isothiocyanate (FITC)-labeled goat F(ab)$'_2$ antihuman immunoglobulin was purchased from Cappel Laboratories, Cochranville, PA. Subset and/or stage-specific surface markers were analyzed by an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) using commercially available monoclonal antibodies (MoAbs) and FITC-labeled F(ab)$'_2$ fragment of goat antimouse IgG antibody (Cappel Laboratories). The MoAbs J-5,[13] OKIa1,[14] OKB1,[15] OKB2,[16] OKT3,[17] OKT4,[17] and OKT8[17] were purchased from Ortho Pharmaceutical, Raritan, NJ. Terminal deoxynucleotidyl transferase (TdT) activity was assayed by the method of Hoffbrand et al[18] and was given as units per 10^8 cells where 1 unit equaled to 1 nmol of substrate polymerized per hour. Epstein-Barr nuclear antigen (EBNA) was assayed by the anticomplement immunofluorescence method as described by Reedman and Klein.[19]

Chromosomal analysis. The cells were cultured in the presence of colcemide for one hour before harvesting, resuspended in 0.075 mol/L KCl for 15 minutes, spread on microscopic slides, fixed in 3:1 methanolacetic acid, and air dried. A G-banding technique was used for analysis.[20]

Analyses of immunoglobulin, T cell β-receptor, and bcr genes. High-mol wt DNA was extracted from the cells according to the method of Perucho et al[21] with minor modifications.[22] Ten to 20 μg of DNA were digested to completion with either *Eco*RI, *Bam*HI, *Hind*III, or *Bgl*II, electrophoresed through 0.75% agarose gels and transferred to nitrocellulose membranes.[23] The membranes were hybridized with nick-translated DNA probes as described previously,[22] washed five times with a solution containing 0.5 × SSC (45 mmol/L sodium chloride plus 4.5 mmol/L sodium citrate) and 0.1% (wt/vol) SDS at 65 °C and autoradiographed at −80 °C with an intensifying screen for several days. J$_H$ probe was a 3.3-kb *Eco*RI-*Hind*III fragment of (Ch4AHIgμ24),[24] and J$_\kappa$ probe was a *Sac*I-*Sac*I fragment of embryonic J$_\kappa$ region,[25] both obtained by the courtesy of Dr T. Honjo.

A plasmid (JUR-β2) containing human Tβ cDNA (JUR-β 2), which spanned from nine bases 5′ of J segment to poly-A tract,[26] was kindly provided by Dr T. W. Mak, and its 0.85-kb *Pst*I fragment was used as Tβ probe. A human *bcr* probe (Probe-1), a 1.2-kb *Hind*III-*Bgl*II fragment,[10] was purchased from Oncogene Science, Mineola, NY.

Analyses of c-abl genes' expression. RNA was extracted by the guanidinum, thiocyanate-cesium chloride method[27] and selected for poly(A)-containing species on an oligo(dT)-cellulose column.[28] Northern blot analysis of polyadenylated RNA extracted from the cells was carried out according to the method of Goldberg.[29] Ten micrograms of polyadenylated RNA in a solution containing 50% formamide; 7% formaldehyde; 20 mmol/L Mops, pH 7.0; 5 mmol/L Na acetate; and 1 mmol/L EDTA were heated to 60 °C for five minutes and cooled immediately on ice. The samples were electrophoresed through a 1.0% agarose gel containing 7% formaldehyde. Then the gel was equilibrated in 20× SSC for 45 minutes and blotted onto Biodyne membrane (Pall, Glen Cove, NY). After

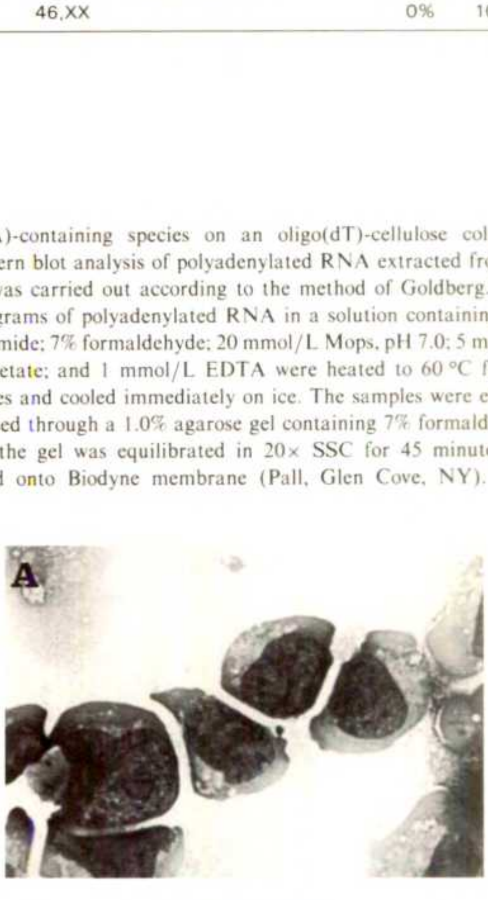

Fig 1. (A) Morphological characteristics of the TOM-1 cells and (B) the original leukemic cells in bone marrow of the patient stained with May-Grünwald-Giemsa.

Table 2. Phenotypical Characterization of the Original Leukemic Cells and the TOM-1 Cells at Various Days After the Beginning of Cultivation

	Patient's Leukemic Cells (Bone Marrow)	TOM-1 Cells					
	11/7/83	2/3/84	4/26/84	8/31/84	12/5/84	2/13/85	10/30/85
J-5	81%	76%	13%	5%	0	0	0
OKIa1	49%	99%	77%	99%	95%	95%	93%
OKB1	5%	69%	64%	70%	90%	95%	90%
smIg	0	0	0	0	NT	0	0
cIg	0	0	0	NT	0	NT	0
OKT3	30%	0	NT	NT	0	NT	NT
OKT4	10%	0	NT	NT	NT	NT	NT
OKT8	20%	0	NT	NT	NT	NT	NT
OKT11	NT	NT	NT	NT	NT	NT	NT
E-R	0	NT	0	NT	NT	NT	NT

Abbreviations: NT, not tested; E-R, E rosette formins cells.

baking in a vacuum at 80 °C for two hours, the membrane was hybridized with 2 to 3 × 10^6 cpm/mL of a nick-translated *abl*-specific probe (1.5-kb *Bgl* II fragment of pAb3[30]) in a solution containing 50% formamide; 5× Denhardt's solution; 5× SSPE; 0.3% SDS; 250 μg/mL *Escherichia coli* DNA at 42 °C overnight; washed three times with 2× SSC, 0.1% SDS and two times with 0.1× SSC, 0.1% SDS; and then autoradiographed at −80 °C.

Cultured leukemic cell lines and fresh leukemic cells used for gene analyses. Two Ph^1-positive leukemic cell lines, K562[7] and NALM-1,[8] and a T cell leukemic cell line, Molt-4F,[31] were used as controls. Fresh leukemic cells of six patients with Ph^1-positive ALL and seven patients with Ph^1-positive CML were obtained from either peripheral blood, bone marrow, or spleen. The clinical profiles and the analysis of cell surface markers of Ph^1-positive ALL cases are summarized in Table 1.

RESULTS

Cultivation of primary leukemic cells and establishment of a cell line. A gradual proliferation of leukemic cells was observed within a few days of culture. One half to one third of the medium was changed weekly. After the fourth week the cells started to grow vigorously and were maintained in a continuous culture by serial cell transfers. This cell line, TOM-1, has been maintained for more than 100 generations.

Cytologic characteristics of TOM-1 cell line. May-Grünwald-Giemsa (MGG) staining showed that the TOM-1 cells were mostly round or ovoid and uniformly large (12 to 15 μm in diameter). Each cell contained an ovoid nucleus composed of fine chromatin with one or two nucleoli. The cytoplasm was remarkably basophilic and contained no granules (Fig 1A). These cells were similar to the original leukemic cells in the patient's bone marrow (Fig 1B). Cytochemical analyses of the TOM-1 cells and the original leukemic cells showed that both cells were positive for PAS stain but negative for peroxidase, SBB, acid phosphatase, a-NBE, and NACE stains.

Phenotypical analysis. The results of surface marker analysis are shown in Table 2. The original leukemic cells were Ia-positive and CALLA-positive but lacked T cell markers and surface and cytoplasmic immunoglobulins. It

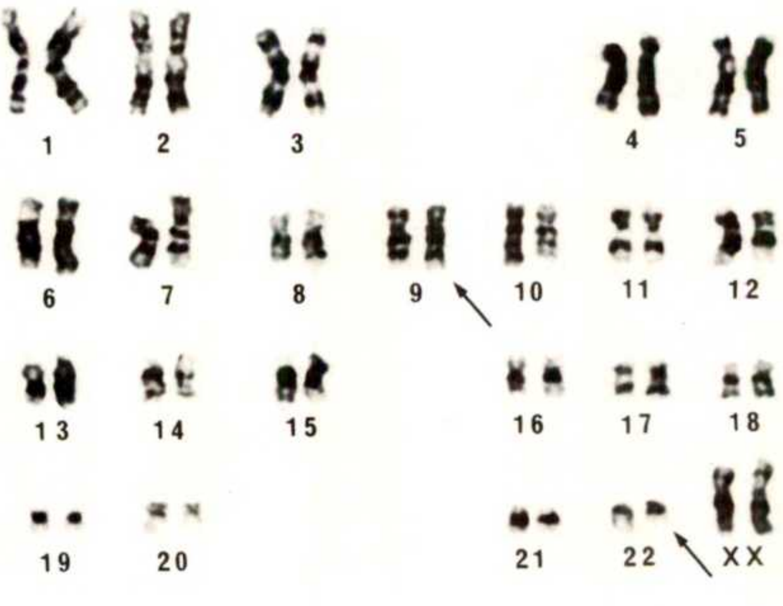

Fig 2. Chromosomal analysis of the TOM-1 cells. The Ph^1 chromosome, resulting from reciprocal translocation of chromosome 9 and 22 t(9:22), is indicated by arrows (→).

was not certain whether they were positive for the B1 antigen, because only 5% of the bone marrow aspirate cells were B1 antigen positive. Three months after the beginning of culture, the TOM-1 cells were 76% CALLA-positive, 99% OKIa1-positive, and 69% B1-positive. Thereafter they tended to lose CALLA-positivism, but the expression of B1 and Ia antigens remained unchanged. Neither surface Ig nor cytoplasmic Ig was detected. TdT activity was positive, and EBNA was negative.

Chromosomal analysis. Repeated karyotype analyses have revealed that all TOM-1 cells had the Ph^1 chromosome. Most cells showed the karyotype 46, XX, t(9:22) (Fig 2), but a few cells showed aneuploid karyotypes (47 to 49). These chromosomal and phenotypical analyses support the derivation of TOM-1 cells from the original leukemic cells.

Immunoglobulin gene configurations. Southern hybridization of *Eco*RI-digested DNA with J_H probe (3.4-kb *Eco*-RI-*Bam*HI fragment) is shown in Fig 3B. The TOM-1 cells contained rearranged J_H genes (lane 1). Southern hybridization of *Eco*RI-digested DNA with J_K probe showed that the TOM-1 cells contained only germ-line J_K genes (Fig 3C).

The leukemic cells from five patients with Ph^1-positive ALL were analyzed for their phenotypic profiles and Ig gene

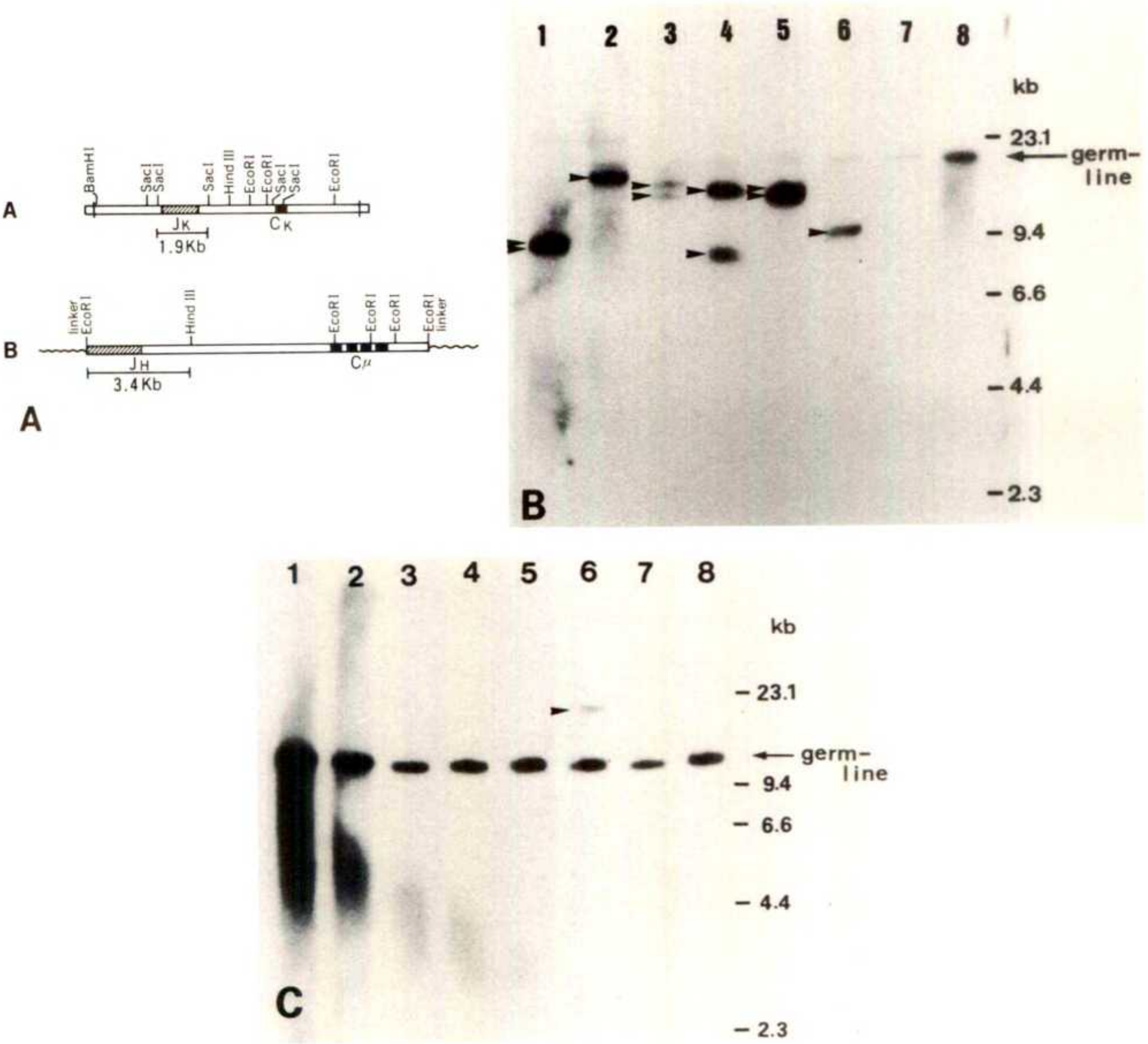

Fig 3. Analysis of immunoglobulin gene configurations by Southern blot hybridization. (A) The human Ig gene probes used in this study. A: restriction map of J_κ probe, a 1.9-kb *SacI-SacI* fragment was used as J_κ probe. B: restriction map of p(Ch4AH Igμ24). 3.4-kb *Eco*-RI-*Hind*III fragment subcloned in pBR 322 was kindly supplied by Dr T. Honjo. The 3.4-kb fragment was purified and used as J_H probe. (B) Configuration of J_H genes in *Eco*RI-digested DNA. (C) Configuration of J_κ genes in *Eco*-RI-digested DNA. The rearranged genes are indicated by arrows (▶). The TOM-1 cells (lane 1) and all five cases of Ph^1-positive ALL (lanes 2 through 6) showed rearranged J_H genes. The Molt-4F, a T cell leukemia cell line (lane 7), showed germ-line J_H gene (or an allele could have been deleted). A case of Ph^1-positive ALL (lane 6) contained a rearranged J_κ gene in *Eco*RI-digested RNA. Human placenta DNA is in lane 8.

rearrangements. The leukemic cells from all cases were Ia$^+$, CALLA$^+$, Pan-B$^{+/-}$, cIg$^-$, sIg$^-$, and T cell markers$^-$. These characteristics are common to CALLA-positive non-T, non-B ALL. All leukemic cells contained rearranged J_H genes (Fig 3B). Four of five cases showed germ-line J_K genes, but one case contained rearranged J_K genes in *Eco*-RI-digested DNA (Fig 3C, lane 6).

T cell β-receptor gene configurations. The Tβ cDNA probe used was JUR-β 2, which hybridizes with Cβ1 and Cβ2 because these two gene segments share a high degree of homology. *Eco*RI-digested germ-line Tβ region genes gave two hybridization signals, 11 kb for Cβ1 and 4 kb for Cβ2 (Fig 4A).[28] *Hind*III-digested germ-line Tβ region genes gave three hybridization bands, 8kb and 6 kb for Cβ2 and 3 kb for Cβ1 (Fig 4B). The TOM-1 cells contained only germ-line Tβ genes (lane 1). In five Ph^1-positive ALL cases, three cases contained germ-line patterns, but two other cases contained rearranged Tβ genes. (They showed deleted Cβ1 genes in both *Eco*RI-digested DNA and *Hind*III-digested DNA and rearranged Cβ2 genes in *Hind*III-digested DNA [lanes 3 and 4]). The results of Ig and Tβ gene analyses are summarized in Table 3.

Analysis of bcr gene rearrangements. We investigated *bcr* gene rearrangements in the TOM-1 cells and Ph^1-positive ALL cells (Fig 5). The TOM-1 cells and the K562 cells contained the rearranged *bcr* gene, although it was not detected in the NALM-1 cells. In three of 6 cases of Ph^1-positive ALL, the breakpoints were within *bcr* (lanes 4, 6, and 9). Case 5 showed the breakpoint near but not within *bcr* because rearrangement was found only in *Eco*RI-digested DNA (lane 8). Two cases showed germ-line pattern (case 2, lane 5; case 4, lane 7). In Ph^1-positive CML cases, all seven cases showed rearrangements within or near *bcr* (only one case was shown in Fig 5) (Table 4). A patient with refractory anemia with an excess of blasts (RAEB) in "transformation" who showed the hematologic findings simi-

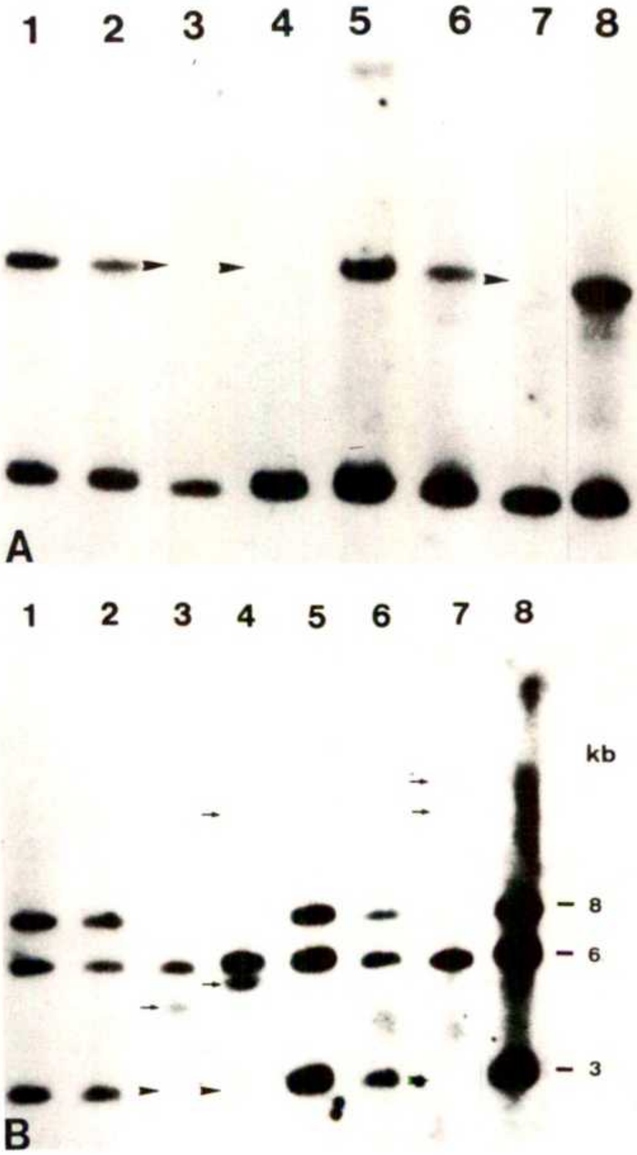

Fig 4. T cell β-receptor gene configurations. (A) Rearrangement patterns of TCβ genes in *Eco*RI-digested DNA and (B) in *Hind*III-digested DNA. Deleted or rearranged genes are indicated by marks (▶) or arrows (→), respectively. The TOM-1 cells and human placenta DNA contain germ-line Cβ genes (lanes 1 and 8, respectively). Those of the leukemic cells from five cases of Ph^1-positive ALL are shown in lanes 2 through 6. Case 2 (lane 3) and case 3 (lane 4) show rearranged Cβ 2 genes.

Table 3. Ig and Tβ Genes' Configurations and Immunophenotypic Profiles in the TOM-1 Cells and the Leukemic Cells of Ph^1-Positive ALL Cases

							Ig Gene Patterns			Tβ Gene Patterns (Cβ_1/Cβ_2)	
	Phenotypical Markers						J$_H$	J$_\kappa$			
Case	Ia	CALLA	Pan-B	Pan-T	cIg	sIg	*Eco*RI	*Eco*RI	*Bam*HI*	*Eco*RI	*Hin*dIII
TOM-1	+	−	+	−	−	−	R/R	G/G	G/G	G/G	G/G
Ph^1-positive ALL cases											
1	+	+	+	−	−	−	R/D	G/G	G/G	G/G	G/G
2	+	+	−	−	−	−	R/R	G/G	G/G	D/G	D/R
3	+	+	+	−	−	−	R/R	G/G	G/G	D/G	D/R
4	+	+	+	−	−	−	R/R	G/G	NT	G/G	G/G
5	+	+	+	−	−	−	R/D	R/G	NT	G/G	G/G

Abbreviations: G, germ-line patterns; R, rearranged patterns; D, deleted pattern; NT, not tested.

*Data not shown.

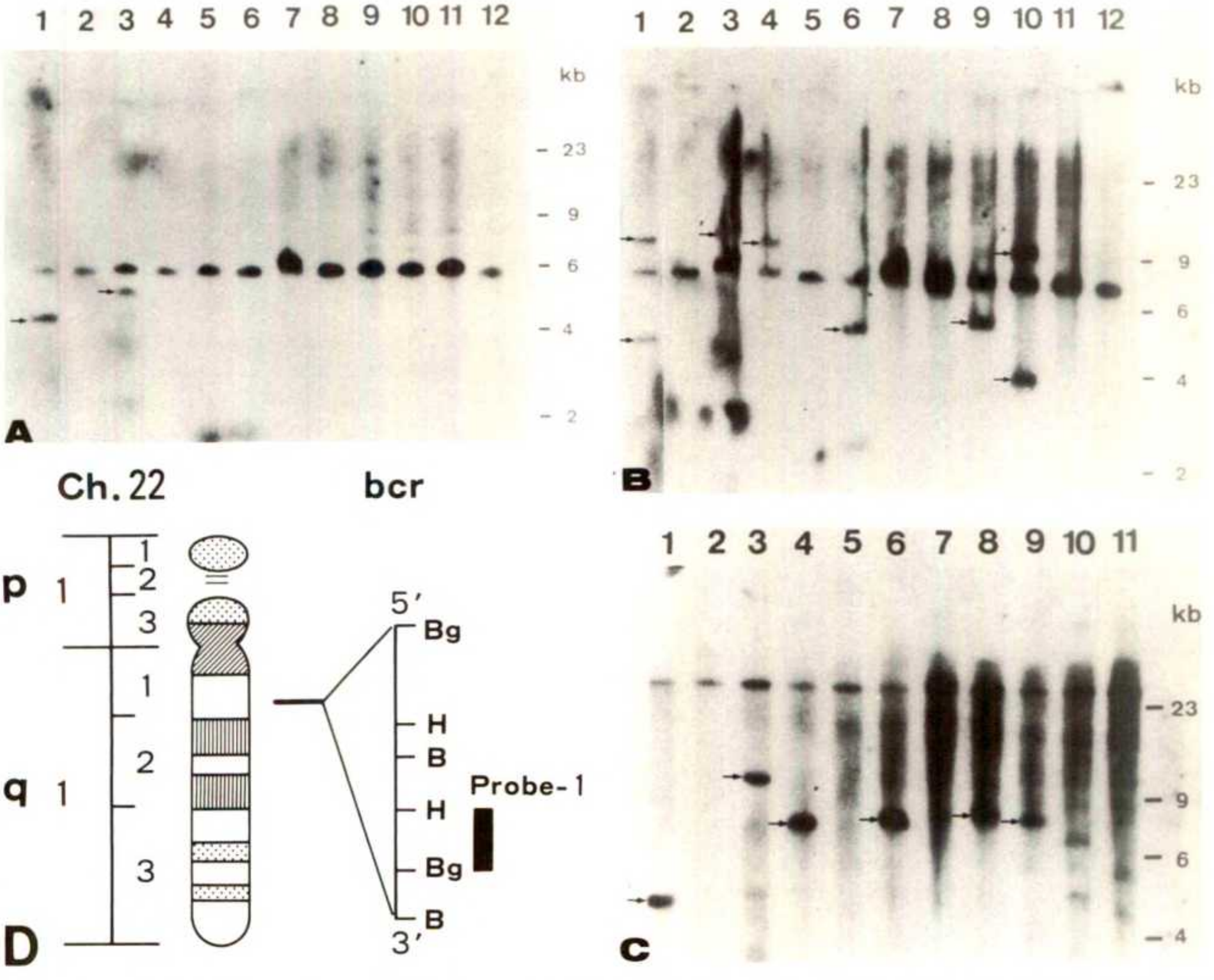

Fig 5. Analysis of *bcr* rearrangements. The analyses were carried out in (A) *Hin*dIII-digested, (B) *Bgl*II-digested, or (C) *Eco*RI-digested DNA. The rearrangements are indicated by arrows (→). Lane 1: the K562 cells; lane 2: the NALM-1 cells; lane 3: the TOM-1 cells; lanes 4 through 9: Ph^1-positive ALL cases; lane 10: CML case; lane 11: RAEB in "transformation" case; lane 12: human placenta DNA. (D) The breakpoint cluster region on chromosome 22. A restriction map of *bcr* gene, of which maximum size is 5.8 kb, and a 1.2-kb *Hin*dIII-*Bgl*II fragment of the human *bcr* probe, Probe-1, are shown, as previously described by Groffer et al.[10] Abbreviations: Bg, *Bgl*II; H, *Hin*dIII; B, *Bam*HI.

Table 4. Analysis of *bcr* Gene Rearrangements

Case	*Hin*dIII	*Bgl*II	*Bam*HI*	*Eco*RI
K562	R	R	R	R
NALM-1	G	G	G	G
TOM-1	R	R	R	R
Ph^{1+} ALL cases				
1	G	R	NT	R
2	G	G		G
3	G	R		R
4	G	G		G
5	G	G		R
6	G	R		R
Ph^{1+} CML cases				
1	G	R	NT	G
2*	R	R		R
3*	G	G		R
4*	G	G		R
5*	G	R		R
6*	R	R		R
7*	R	R		R
RAEB in "T" case	G	G	NT	G

Abbreviations: R, rearranged; G, germ-line pattern.

*Data not shown.

lar to those of Ph^1-negative CML (lane 11) and human placenta DNA (lane 12) showed germ-line patterns. These results are summarized in Table 4.

c-abl Gene expression. Amplification of c-*abl* gene in the TOM-1 cells and Ph^1-positive ALL cells was not detected by Southern blot analysis (data not shown). Northern blot analysis of polyadenylated RNA showed that the TOM-1 cells contained *abl*-related mRNA of only normal length, approximately 6-kb and 4-kb bands, without 8 kb transcript. On the other hand, the leukemic cells isolated from a patient with CML in myeloid crisis contained a novel 8-kb *abl*-related mRNA in addition to transcripts of normal length (Fig 6).

DISCUSSION

The Ph^1 chromosome, resulting from reciprocal translocations between chromosomes 9 and 22, t(9:22), is a cytogenetic abnormality found in more than 90% of CML patients.[1] Ph^1-positive leukemia cells have also been found in patients without history of chronic CML. Some of them show the features of acute myelogenous leukemias, but others clearly show those of ALL. Two series of investigations suggested an incidence of about 25% Ph^1-positivism among adult ALL cases,[2,3] and thus Ph^1-positive leukemia cells are not rare among adult ALL. However, as reported by Beard et al,[32] a blastic crisis of CML could occur without a preceding chronic phase. Therefore it may be necessary to differentiate lymphoid crisis of CML from Ph^1-positive ALL.

The patient presented herein, from whose bone marrow the TOM-1 cell line was derived, showed features of Ph^1-positive ALL. The Ph^1 chromosome was identified in the bone marrow cells on admission but disappeared from both bone marrow and peripheral blood cells once complete remission was achieved. Chromosomal analysis also showed no additional abnormality except for the presence of the Ph^1 chromosome, which is frequently found in blastic crisis of CML. Furthermore, a conversion into chronic CML was not seen. As these results support that the Ph^1 chromosome existed in the lymphoid cells but not in the hematopoietic stem cells, the diagnosis of Ph^1-positive ALL was appropriate.

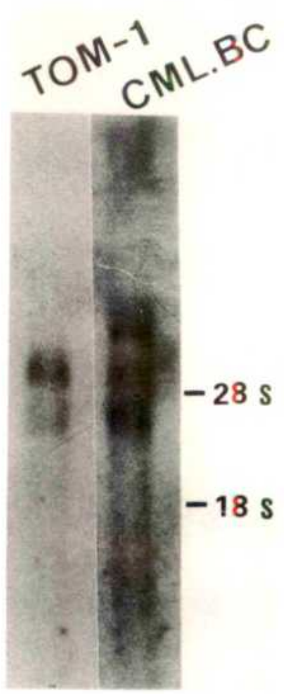

Fig 6. *abl*-Related mRNA expression. Northern blot analysis of polyadenylated RNA showed the TOM-1 cells contained *abl*-related mRNA of normal length, 6 kb and 4 kb bands; nevertheless, a case of CML in myeloid crisis (CML.BC) contained 8-kb *abl*-related mRNA in addition to transcripts of normal sizes.

The TOM-1 cells derived from this patient were positive for Ia and B1 antigens and were negative for surface and cytoplasmic Ig, CALLA, and T cell markers. Korsmeyer et al found that Ig gene rearrangements occurred in non-T, non-B (no cytoplasmic Ig)-cell ALL and concluded that most leukemic cells of non-T, non-B ALL resided as the progenitor of pre-B along the B cell pathway where they initiated J_H gene rearrangement, bore surface HLA-DR antigens, and later acquired CALLA and BA-1 antigens.[33] The TOM-1 cells contained rearranged IgH chain genes but retained germ-line κ chain and germ-line Tβ genes. These findings indicate that the TOM-1 cells reside at the progenitor level of pre-B cells.

Molecular events accompanied by the t(9:22) translocation have been extensively analyzed for Ph^1-positive CML, especially K562 cell line.[8,9] A novel 8-kb c-*abl*-related mRNA specific to CML is a fused transcript of two genes, the breakpoint cluster region gene on chromosome 22 and the c-*abl* gene translocated from chromosome 9 to 22.[34] The fused protein would unmask protein kinase activity of c-*abl* protein and may be important in the leukemic process of CML.[35] Recently Rodenhuis et al described that Ph^1 chromosome of childhood ALL does not involve *bcr*, in sharp contrast to Ph^1-positive CML.[36] In the present study we found that all seven cases of CML had a breakpoint within or near *bcr*, that TOM-1 cells also showed the breakpoint within *bcr*, and that four of six adult Ph^1-positive ALL cases contained rearranged *bcr* genes. We suggest the possibility that the breakpoints on chromosome 22 of adult Ph^1-positive

ALL might be different from those of childhood Ph^1-positive ALL.

By Southern blot analysis c-*abl* gene amplification in the TOM-1 cells and in the leukemic cells of Ph^1-positive ALL patients were not found. Northern blot analysis showed that 8-kb c-*abl*-related mRNA, in addition to the normal transcripts (6 kb and 4 kb), was found in the leukemic cells of Ph^1-positive CML in a myeloid crisis patient. On the other hand, the TOM-1 cells contained c-*abl* gene transcripts of only normal length, despite the fact that they showed the *bcr* gene rearrangement. At present, whether the observed c-*abl* gene expression in the TOM-1 cells is the representative of Ph^1-positive ALL accompanied by the *bcr* gene rearrangements remains to be determined; nevertheless, this finding suggests the possibility that the genetic mechanism in Ph^1-positive ALL might be different from that reported for CML. The newly developed TOM-1 cell line may be beneficial to this line of investigation.

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Impaired Nicotinamide Adenine Dinucleotide Synthesis in Pyruvate Kinase-Deficient Human Erythrocytes: A Mechanism for Decreased Total NAD Content and a Possible Secondary Cause of Hemolysis

By Charles R. Zerez and Kouichi R. Tanaka

Erythrocytes from individuals with pyruvate kinase (PK) deficiency have approximately half the total (oxidized and reduced) nicotinamide adenine dinucleotide (NAD) of normal erythrocytes. In order to elucidate the mechanism(s) for the decrease in total NAD, we examined NAD synthesis in intact erythrocytes. It is demonstrated that NAD synthesis is impaired in PK-deficient erythrocytes to a degree that is dependent on the PK activity and adenosine 5′-triphosphate (ATP) concentration of these cells. After incubation in the presence of fluoride, which simulates the characteristics of PK deficiency by inhibiting enolase, normal erythrocytes had impaired NAD synthesis and decreased ATP concentrations. Fluoride did not inhibit NAD synthesis in a hemolysate system that is not dependent on glycolysis for ATP generation. These data suggest that fluoride does not inhibit the enzymes of NAD synthesis and that impairment of NAD synthesis by fluoride is mediated by decreased ATP formation. Thus, it is concluded that impaired NAD synthesis in PK-deficient erythrocytes is caused by decreased ATP formation due to the PK deficiency. Since the rate of glycolysis is limited by the availability of NAD$^+$, it is suggested that impaired NAD synthesis causes further ATP depletion and thereby may enhance hemolysis in PK-deficient erythrocytes.

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ERYTHROCYTES from pyruvate kinase (PK)-deficient individuals are characterized by several metabolic abnormalities.[1] One abnormality that remains unexplained is the decreased concentration of total (oxidized and reduced) nicotinamide adenine dinucleotide (NAD) in these cells.[2,3] Since the total NAD concentration in normal erythrocytes is approximately 40 μmol/L[4] and the K_m for NAD$^+$ of glyceraldehyde-3-phosphate dehydrogenase (G3PD) under physiological conditions is 143 μmol/L,[5] the rate of the G3PD reaction in vivo is limited by the concentration of NAD$^+$. Therefore, PK-deficient erythrocytes, with approximately half the total NAD of normal erythrocytes,[2,3] should have a lower in vivo G3PD reaction rate than normal erythrocytes. This is significant because, at steady state, a decreased rate of flux through G3PD will also cause a decreased rate of flux through the glycolytic reactions that follow the G3PD reaction. This includes both the phosphoglycerate kinase reaction, immediately following the G3PD reaction, and the PK reaction, which are the only adenosine 5′-triphosphate (ATP)-generating reactions in mature erythrocytes. Thus, decreased flux through the glycolytic reactions following the G3PD reaction will result in decreased ATP regeneration, which may enhance the hemolytic process in PK-deficient erythrocytes.

The decrease in the total NAD concentration in PK-deficient erythrocytes cannot be attributed to a decreased rate of flux through lactate dehydrogenase since the concentrations of both the oxidized and reduced forms of NAD are decreased. Thus, a decreased total NAD concentration must be due to other metabolic processes such as changes in the rate of synthesis or degradation of this coenzyme. It is not clear how intraerythrocytic NAD is degraded in humans since the only known enzyme capable of NAD degradation, NAD glycohydrolase (NADase; EC 3.2.2.6), is known to be located on the exterior of the erythrocyte membrane with no access to intraerythrocytic NAD.[6,7] NADase has also been shown to be an extracellular membrane-bound enzyme in other mammalian erythrocytes.[8,9] Therefore, to determine the mechanism responsible for the decrease in the total NAD concentration in PK-deficient erythrocytes, we have concentrated our efforts on an examination of the capacity of these cells to synthesize NAD. Human erythrocytes are capable of NAD synthesis from nicotinic acid (niacin) in a three-reaction pathway first elucidated by Preiss and Handler[10,11] (Fig 1). NAD biosynthesis is highly dependent on the availability of ATP: in addition to the ATP required for NAD biosynthesis from nicotinic acid and 5-phosphoribosyl-1-pyrophosphate (PRPP) (Fig 1), ATP is also required for the synthesis of PRPP from ribose-5-phosphate.[12] The data presented in this paper suggest that the decrease in the total NAD concentration in PK-deficient erythrocytes is due to impaired NAD synthesis caused by decreased ATP regeneration.

MATERIALS AND METHODS

Materials

D-Glucose was purchased from J.T. Baker Chemical Co, Phillipsburg, NJ. All other reagents were purchased from Sigma Chemical Co, St Louis. The potassium salt of fluoride was used throughout this investigation.

Methods

Isolation of erythrocytes. After obtaining informed consent, blood was obtained by routine venipuncture from PK-deficient

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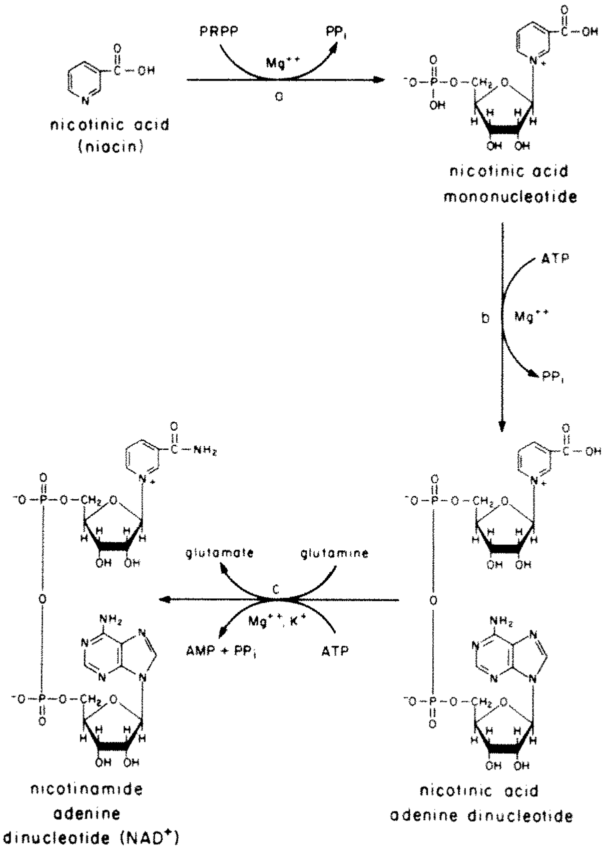


Fig 1. NAD biosynthesis in human erythrocytes. The reactions shown are catalyzed by nicotinic acid phosphoribosyltransferase (EC 2.4.2.11), reaction a; nicotinic acid adenine dinucleotide pyrophosphorylase (EC 2.7.7.18), reaction b; and NAD synthetase (EC 6.3.5.1), reaction c. AMP, adenosine 5′-monophosphate; PP$_i$, inorganic pyrophosphate.

individuals and anticoagulated with heparin (15 U/mL whole blood). Blood from normal subjects and from individuals with hemolytic anemia of unknown etiology served as appropriate controls. The separation and washing of erythrocytes have been described previously.[13] Erythrocytes were used immediately after isolation.

Preparation of hemolysates. Hemolysates used to determine PK activity were prepared by using a freeze-thaw method.[13] Hemolysates used to measure NAD synthesis were prepared by using osmotic lysis of intact cells without freeze-thawing since the latter manipulation markedly decreases the conversion of nicotinic acid to the NAD precursor nicotinic acid mononucleotide[14] (Fig 1, reaction a). Suspensions of erythrocytes were centrifuged at 3,000 g for ten minutes. Two volumes of a solution containing 2.7 mmol/L EDTA, pH 7.0, and 0.70 mmol/L 2-mercaptoethanol were added to the packed erythrocytes and mixed gently. Examination under a light microscope revealed that more than 99.9% of the erythrocytes were lysed after this treatment. This hemolysate was used immediately in the NAD synthesis assay.

NAD synthesis assay. Conditions for the NAD synthesis assay in intact erythrocytes were a modification of the system of Preiss and Handler.[10] The incubation mixture contained 40 μmol potassium phosphate, pH 7.4; 75 μmol KCl; 75 μmol NaCl; 30 μmol D-glucose; 1.0 μmol nicotinic acid; 5.0 μmol L-glutamine; and intact erythrocytes equivalent to 42 mg hemoglobin (Hb) in a total volume of 1.00 mL. The assay was started by adding the appropriate volume of erythrocyte suspension, and the complete incubation mixture was maintained at 37°C. Conditions for the NAD synthesis assay in hemolysate were developed by modifying the previous incubation mixture. Glucose and inorganic phosphate were replaced with PRPP so that NAD synthesis would be independent of glycolysis. NaCl was eliminated since the maintenance of isotonic conditions was no longer necessary, and $MgCl_2$ was added since the endogenous concentration of magnesium is, presumably, decreased after cell lysis due to dilution of the cell contents. Thus, the incubation mixture for NAD synthesis in hemolysate contained 10 μmol Tris-HCl, pH 7.4; 5.0 μmol KCl; 1.0 μmol nicotinic acid; 15 μmol $MgCl_2$; 5.0 μmol PRPP; 5.0 μmol ATP; 5.0 μmol L-glutamine; and hemolysate equivalent to 42 mg Hb in a total volume of 1.00 mL. The assay was started by adding the appropriate volume of hemolysate, and the complete incubation mixture was maintained at 37°C.

NAD synthesis in both the intact erythrocyte and hemolysate incubation mixtures was determined by measuring the accumulation of the total (oxidized and reduced) NAD concentration after various times of incubation. Thus, at the times indicated, a 0.20-mL aliquot was withdrawn, maintained in a boiling water bath for 60 seconds, and then cooled to 0°C. The heat-treated aliquot was centrifuged to remove cell debris and denatured proteins. To minimize interference by compounds present in the incubation mixture, the supernatant was diluted tenfold before determination of the total NAD concentration with the sensitive cycling method of Bernofsky and Swan.[15] The total NAD concentration, instead of oxidized NAD (NAD$^+$), was measured since NAD$^+$ can readily be converted to its reduced form (NADH) following synthesis.

ATP formation from PRPP. Conditions for ATP formation from PRPP and endogenous AMP and inorganic phosphate were identical to those used to measure NAD synthesis in the hemolysate

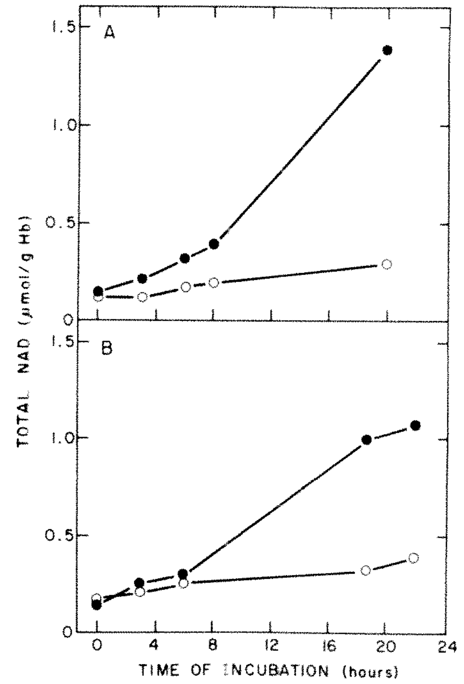


Fig 2. NAD synthesis in intact normal erythrocytes. (A) Optimal conditions were used (see Materials and Methods). (B) Physiological conditions were used (5.0 mmol/L glucose, 1.0 mmol/L glutamine, and 5.0 mmol/L inorganic phosphate; other conditions are the same as for the optimal system). The following incubation conditions were used: complete system (closed circles), without glutamine (open circles).

Table 1. The Quantity of NAD Synthesized in Intact Red Cells From Normal Subjects and PK-Deficient Patients After 20 Hours of Incubation

Subject	Splenectomy	PK Activity (μmol/min · mL RBC)	ATP (μmol/g Hb)	Total NAD Accumulated (μmol/g Hb)
Normal (n = 8)	No	5.53 ± 0.82	3.61 ± 0.66	1.35 ± 0.24
PK deficient				
Patient 1 (41% reticulocytes)	Yes	0.60	1.90	0.53
Patient 2 (7.5% reticulocytes)	No	0.57	2.54	0.70
Patient 3 (7.5% reticulocytes)	No	1.52	2.83	1.04

system (see earlier) except that ATP was omitted from the reaction mixture. When ATP formation from PRPP was measured in the presence of exogenous AMP and inorganic phosphate, the aforementioned reaction mixture was supplemented with 1.0 mmol/L AMP and 20 mmol/L potassium phosphate (pH 7.4). At the indicated times, aliquots were withdrawn and heat-treated as described earlier. Following centrifugation, the supernatant was used to quantitate ATP as described by Beutler.[16]

The rate of ATP formation was also measured by using a continuous spectrophotometric assay that contained 10 μmol Tris-HCl, pH 7.4; 5.0 μmol KCl; 1.0 μmol nicotinic acid; 15 μmol $MgCl_2$; 5.0 μmol PRPP; 5.0 μmol L-glutamine; 0.40 μmol NADP; 1.0 μmol D-glucose; 1.2 IU glucose-6-phosphate dehydrogenase; 8.0 IU hexokinase; and hemolysate equivalent to 1.7 mg Hb in a total volume of 1.00 mL. The reaction mixture was preincubated at 37°C and then started by adding the appropriate volume of PRPP. The absorbance was monitored at a wavelength of 340 nm by using a Gilford model 252 spectrophotometer (Gilford, Ciba-Corning Diagnostics, Oberlin, OH) with a slit width of 0.80 mm. The relatively wide slit width was required because the amount of hemolysate necessary for the reaction resulted in a relatively high initial absorbance. However, because the absorbance band of NADPH is fairly wide, the millimolar extinction coefficient of NADPH remains 6.22 at a slit width of up to 1.0 mm.[16]

Other methods. PK activity was determined as described by Tanaka.[17] The whole blood ATP content was determined spectrophotometrically[18] by using neutralized perchloric acid extracts.[16] This method was also used to measure the ATP concentration in intact erythrocytes during incubation with fluoride. The Hb concentration was determined by using the cyanmethemoglobin method.[16] Data were expressed as the mean ± 1 SD.

RESULTS

Intact human erythrocytes are capable of NAD synthesis when incubated in the presence of glucose, inorganic phosphate, glutamine, and nicotinic acid.[10,19] We have found that normal erythrocytes increased their original total NAD content five to 15 times after incubation with the latter compounds for 20 hours (Fig 2A). Relatively little total NAD was accumulated in the absence of glutamine from the incubation mixture (Fig 2A). Since glutamine is essential for NAD synthesis in human erythrocytes,[11] this suggests that the total NAD accumulation is due to NAD synthesis. To a lesser extent, the total NAD also accumulated when the concentrations of glucose, glutamine, and inorganic phosphate in the incubation mixture were reduced to simulate more physiological conditions (Fig 2B). This suggests that NAD synthesis in normal mature RBC is physiologically significant.

The quantity of total NAD accumulated after 20 hours of incubation in erythrocytes from eight normal subjects was 1.35 ± 0.24 μmoL/g Hb (Table 1). RBC from five patients with hemolytic anemia of unknown etiology (9.0% to 15% reticulocytes) accumulated 1.14 ± 0.14 μmol NAD/g Hb. In contrast, erythrocytes from three PK-deficient patients accumulated substantially less total NAD than erythrocytes from normal subjects (Table 1). NAD synthesis in PK-deficient erythrocytes correlated positively with PK activity and the level of intraerythrocytic ATP. Erythrocytes from PK-deficient patient 3, who had the highest PK activity and ATP content, accumulated 1.04 μmol total NAD/g Hb (Table 1). In contrast, erythrocytes from PK-deficient patient 1, who had the lowest ATP content, accumulated only 0.53 μmol total NAD/g Hb (Table 1). Since this patient had undergone splenectomy (Table 1), presumably a greater proportion of more defective erythrocytes was in circulation.

Since the capacity of PK-deficient erythrocytes to synthesize NAD correlated with their ATP content, we examined the total NAD accumulation in normal erythrocytes using fluoride to inhibit enolase[20] and reduce ATP formation. Incubation of intact normal erythrocytes with fluoride under conditions that yield optimal NAD synthesis resulted in ATP depletion to a degree that was dependent on the concentration of fluoride added to the incubation mixture (Fig 3). Hence, as little as 0.1 mmol/L fluoride led to a 28% decrease in the ATP content after six hours of incubation (Fig 3). A fluoride concentration of 1.0 mmol/L was sufficient to cause a 34% decrease in the ATP concentration after only three hours of incubation (Fig 3). The addition of fluoride to the incubation mixture resulted in marked inhibition of NAD synthesis in normal intact erythrocytes. A fluoride concentration of only 0.10 mmol/L caused a 17% inhibition of total NAD accumulation (Fig 4). Higher concentrations of fluoride were more effective in impairing NAD synthesis, with 1.0 mmol/L fluoride causing full inhibition of total NAD accumulation (Fig 4).

The impairment of NAD synthesis by fluoride is most likely due to decreased ATP regeneration. However, since it is not known what effect fluoride has on the enzymes of NAD synthesis, we determined the effect of fluoride on these enzymes by measuring total NAD accumulation in a hemolysate system that is not dependent on glycolysis (and enolase).

Since the present experiment is the first known demonstration of NAD synthesis in a human hemolysate system, the

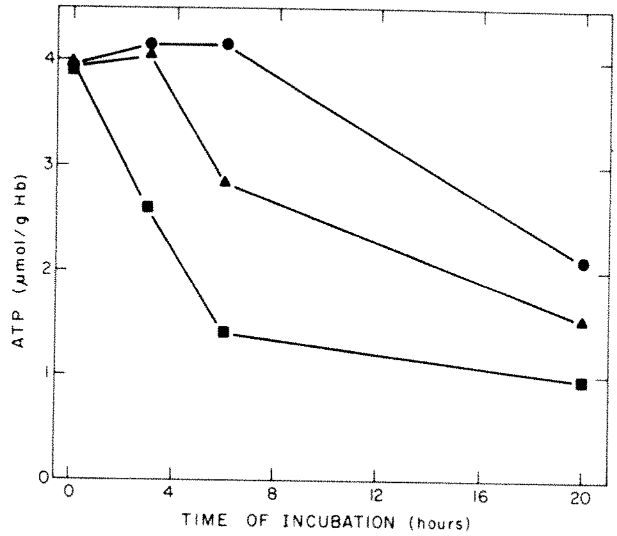


Fig 3. The effect of fluoride on the ATP concentration of intact normal erythrocytes under optimal conditions for NAD synthesis. The following incubation conditions were used: complete system (circles), with 0.10 mmol/L fluoride (triangles), with 1.0 mmol/L fluoride (squares).

Table 2. The Effect of Omissions of Various Components of the Incubation Mixture on NAD Synthesis in Hemolysate

Contents of Incubation Mixture	Total NAD Accumulated in 20 Hours (μmol/g Hb)
Complete	0.361
Without nicotinic acid	0
Without $MgCl_2$	0
Without glutamine	0
Without PRPP	0
Without ATP	0.247
Without ATP and PRPP	0

effect of omissions of various components of the incubation mixture was examined. There was no total NAD accumulation in the absence of nicotinic acid, $MgCl_2$, glutamine, or PRPP after 20 hours of incubation (Table 2). In contrast, the absence of ATP resulted only in a 32% decrease in the total NAD accumulation after 20 hours of incubation (Table 2). However, there was no total NAD accumulation when both ATP and PRPP were excluded from the incubation mixture (Table 2). This suggests that the substantial accumulation of total NAD observed in the absence of added ATP may be due to ATP regeneration from PRPP and endogenous AMP. Previous studies have shown that PRPP synthetase (EC 2.7.6.1), the enzyme responsible for PRPP synthesis from ATP and ribose-5-phosphate, can catalyze the reverse reaction nearly as well as the forward reaction.[21] To determine whether ATP was being regenerated from PRPP under our experimental conditions, we measured ATP formation under the same conditions that yield optimal NAD synthesis in our hemolysate system. To ensure that the presence of the NAD precursors does not interfere with the reverse PRPP synthetase reaction, we first examined ATP formation from PRPP when AMP and inorganic phosphate were exogenously supplied to the reaction mixture. The results indicate that ATP accumulated in a time-dependent manner in the presence of PRPP (Fig 5A). No ATP was found to accumulate when PRPP was absent from the reaction mixture (Fig 5A). This suggests that the NAD precursors present in the reaction mixture do not interfere with ATP formation from PRPP.

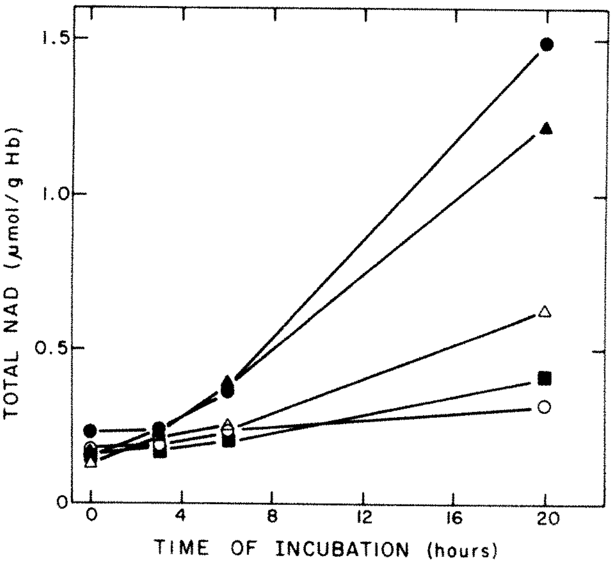
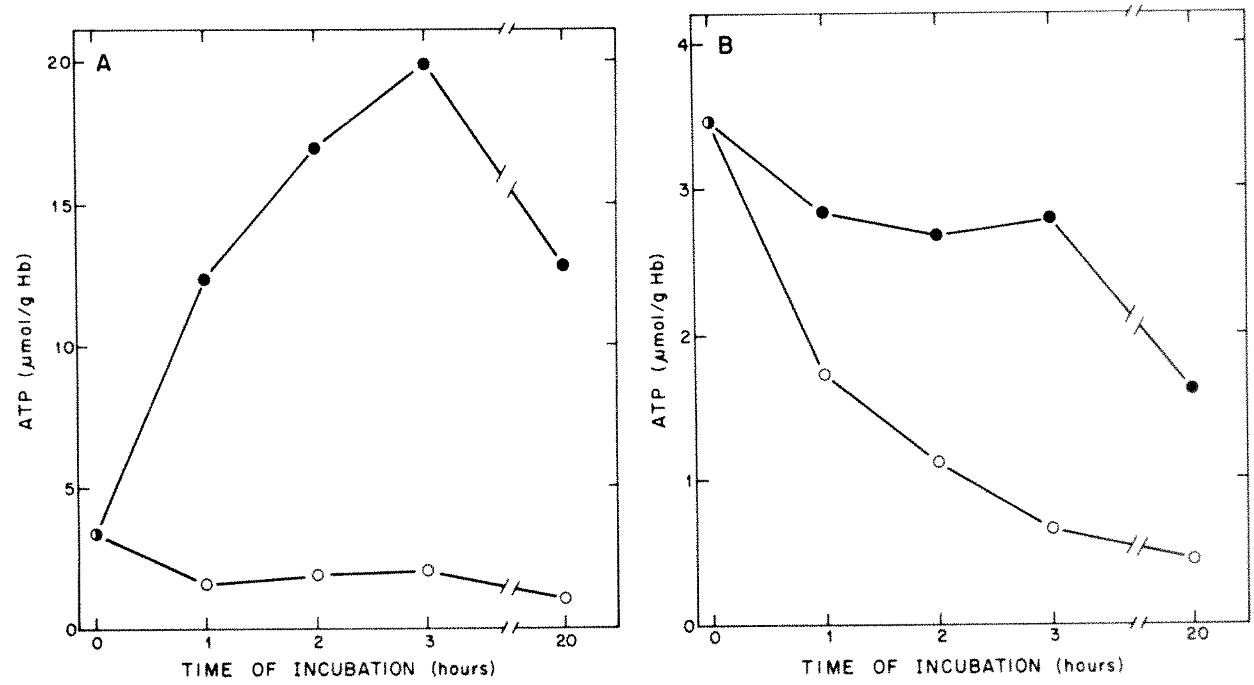


Fig 4. The effect of fluoride on NAD synthesis in intact normal erythrocytes under optimal conditions. The following incubation conditions were used: complete system (closed circles), without glutamine (open circles), with 0.10 mmol/L fluoride (closed triangles), with 0.50 mmol/L fluoride (open triangles), with 1.0 mmol/L fluoride (squares).

Subsequently, we examined ATP formation from PRPP using the hemolysate's endogenous AMP and inorganic phosphate. Under these conditions, no accumulation of ATP was observed in the presence of PRPP (Fig 5B). However, in the absence of PRPP, the ATP content decreased rapidly (Fig 5B). The lack of ATP accumulation in this system suggested that the rate of ATP formation from PRPP and endogenous AMP and inorganic phosphate was not rapid enough to overcome the rate of ATP utilization. To confirm this hypothesis, we devised a continuous spectrophotometric system in which the formation of ATP results in the production of NADPH and a concomitant increase in the absorbance at 340 nm. In this system, the ATP that is formed is "trapped" by the detection system before it can be utilized for NAD synthesis and/or other processes. Under these conditions, ATP was formed readily from endogenous AMP and inorganic phosphate in the presence of PRPP and hemolysate (Fig 5C). It is noteworthy that a significant rate of ATP formation (4.6 μmol/h · g Hb at steady state) was obtained with only 1/25th the hemolysate (1.7 mg Hb) used for the NAD synthesis assay (Fig 5C). No significant ATP formation was observed in the absence of either PRPP or hemolysate (Fig 5C). Furthermore, the addition of exogenous AMP and inorganic phosphate to this system yielded a more rapid rate of ATP formation (Fig 5C) that is consistent with the rapid ATP accumulation of Fig 5A. These data confirm that ATP is formed from PRPP and endogenous AMP and inorganic phosphate under optimal conditions for



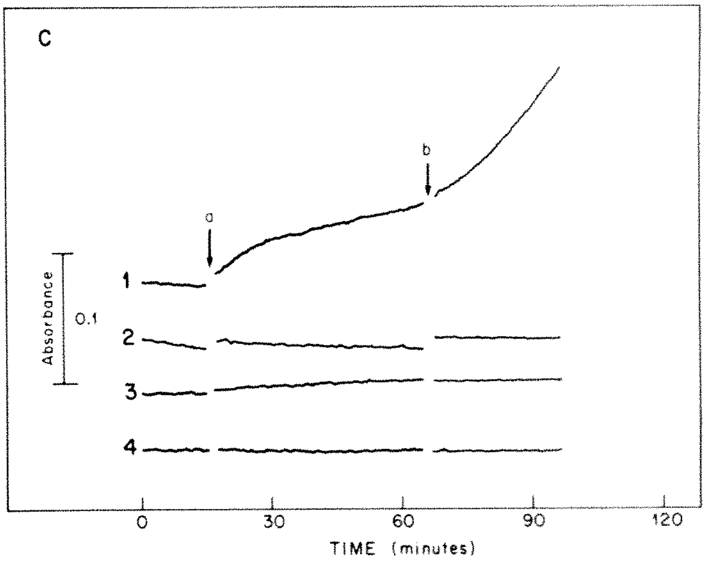


Fig 5. ATP formation from PRPP in hemolysate from normal erythrocytes. Conditions were identical to those used to measure NAD synthesis in hemolysate except that ATP was omitted. (A) Reaction mixtures contained exogenous AMP and inorganic phosphate (see Materials and Methods). (B) Reaction mixtures contained no exogenous AMP and inorganic phosphate. The following incubation conditions were used: complete system (closed circles), without PRPP (open circles). (C) ATP formation measured using a continuous spectrophotometric assay system (see Materials and Methods). The following incubation conditions were used: complete system (tracing 1), without PRPP (tracing 2), without hemolysate (tracing 3), without PRPP and hemolysate (tracing 4). The reactions were started (arrow a) by the addition of PRPP (tracings 1 and 3) or water (tracings 2 and 4). At the indicated time (arrow b), the reaction mixtures were supplemented with AMP and inorganic phosphate to a final concentration of 1.0 mmol/L and 20 mmol/L, respectively.

NAD synthesis in hemolysate. Thus, the substantial quantities of total NAD accumulated in the absence of ATP and in the presence of PRPP (Table 2) is due to the regeneration of ATP from PRPP.

In the hemolysate–NAD synthesis system, there was an initial decrease in the total NAD concentration within the first two hours of incubation (Fig 6). Subsequently, total NAD accumulated at a linear rate up to 20 hours of incubation (Fig 6). The addition of up to 1.0 mmol/L fluoride to the incubation mixture did not affect total NAD accumulation in the hemolysate system (Fig 6). The initial decrease in total NAD concentration that is observed in this system (Fig 6) may be caused by the exposure of the intraerythrocytic NAD pool to the extracellular membrane-bound NADase[6,7] after red cell lysis. The lack of a decrease in the total NAD content when intact erythrocytes are used (Figs 2A and 2B) is consistent with this hypothesis since intraerythrocytic NAD is not exposed to NADase under these conditions.

DISCUSSION

We have shown that intact PK-deficient erythrocytes have impaired NAD synthesis since they accumulated less total NAD than normal erythrocytes when incubated with precursors essential for NAD biosynthesis (Table 1). Impaired NAD synthesis in PK-deficient red cells is not due to the higher proportion of reticulocytes since high reticulocyte red cell samples without PK deficiency did not demonstrate significantly impaired NAD synthesis. The positive correlation between NAD synthesis, PK activity, and the level of

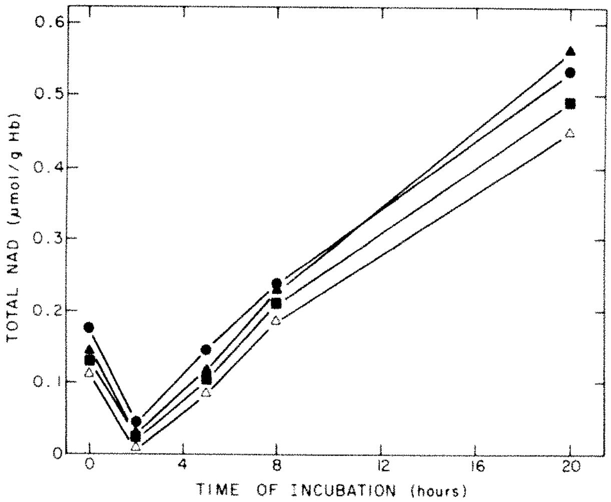


Fig 6. The effect of fluoride on NAD synthesis in hemolysate from normal erythrocytes. The following incubation conditions were used: complete system (closed circles), with 0.10 mmol/L fluoride (closed triangles), with 0.50 mmol/L fluoride (open triangles), with 1.0 mmol/L fluoride (squares).

intraerythrocytic ATP suggested that impaired NAD synthesis in PK-deficient erythrocytes may be caused by decreased ATP regeneration due to low PK activity. To provide further evidence for this hypothesis, we measured total NAD accumulation in normal erythrocytes in the presence of fluoride. This ion is an inhibitor of enolase[20] and has been used previously to simulate the characteristics of PK deficiency.[22] The decrease in the ATP concentration with time at all fluoride concentrations used (Fig 3) suggests that fluoride was inhibiting enolase (and glycolysis) and, presumably, simulating PK deficiency under the conditions used.

We have found that fluoride is a potent inhibitor of NAD synthesis in intact erythrocytes (Fig 4) even at concentrations that cause only partial depletion of ATP (Fig 3). The sensitivity of NAD synthesis to inhibition by fluoride may be due to the requirement of several ATP molecules for the synthesis of one NAD molecule (Fig 1). The inhibition of NAD synthesis in intact erythrocytes by fluoride cannot be due to inhibition of PRPP synthetase since fluoride concentrations as high as 50 mmol/L do not affect PRPP synthetase activity in crude hemolysate.[23] To determine whether fluoride interfered with the activity of the enzymes of NAD biosynthesis, we measured the total NAD accumulation in hemolysate by substituting PRPP for glucose and inorganic phosphate. Under these conditions, NAD synthesis will not be dependent on glycolysis and hence enolase to provide ATP for PRPP formation. There was a substantial accumulation of total NAD in this hemolysate system (Table 2 and Fig 6). Since there was no accumulation of total NAD when components essential for NAD synthesis such as nicotinic acid, glutamine, $MgCl_2$, or PRPP were excluded (Table 2), it is suggested that the total NAD accumulation in the hemolysate system was due to NAD synthesis. The substantial accumulation of total NAD observed in the absence of added ATP is due to ATP regeneration from PRPP and endogenous AMP since no total NAD accumulation is observed in the absence of both ATP and PRPP (Table 2) and since ATP formation from PRPP and endogenous AMP can be readily demonstrated (Fig 5C). When NAD synthesis was measured in the hemolysate system and glycolysis and enolase were bypassed, fluoride had no significant effect on total NAD accumulation (Fig 6). This suggests that fluoride did not inhibit the enzymes of NAD biosynthesis in hemolysate.

The lack of inhibition of the NAD biosynthetic enzymes and PRPP synthetase by fluoride in hemolysate suggests that impairment of NAD synthesis in intact normal erythrocytes in the presence of fluoride is caused by decreased ATP formation. These data support the hypothesis that impaired NAD synthesis in intact PK-deficient erythrocytes is caused by decreased ATP regeneration due to the PK deficiency itself. Furthermore, our data suggest that impaired NAD synthesis is a primary cause of the decreased total NAD concentration in PK-deficient erythrocytes. Previous studies from this laboratory have shown that PK-deficient erythrocytes have decreased PRPP synthetase subunit aggregation and therefore a less active enzyme in vivo.[24] This is caused by the decreased ATP and increased 2,3-diphosphoglycerate concentrations in these erythrocytes.[24] Thus, we have suggested that impaired NAD synthesis may also be mediated by the decreased in vivo activity of PRPP synthetase.[24]

Impaired NAD synthesis in PK-deficient erythrocytes is significant because the rate of the G3PD reaction and hence glycolysis is limited by the NAD^+ content of the erythrocyte. Thus, impaired NAD synthesis may cause further ATP depletion and may be a secondary cause of hemolysis in PK-deficient erythrocytes.

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On the Mechanisms of Sensitization and Attachment of Antibodies to RBC in Drug-Induced Immune Hemolytic Anemia

By A. Salama and C. Mueller-Eckhardt

The mechanisms of sensitization and attachment of drug-dependent antibodies to RBC in drug-induced immune hemolytic anemias are largely speculative. Nomifensine has been incriminated in causing immune hemolysis in a large number of patients. The hemolysis was usually of the so-called immune complex type, less commonly of the autoimmune type, and more surprisingly, few patients had developed both types of hemolysis. To determine whether nomifensine (metabolite)-dependent antibodies (ndab) exhibit specificity for antigenic structures of RBC membranes, 30 ndab were tested against large panels of RBC with common and rare antigens. We found that only 14 out of 30 ndab were invariably reactive with all cells tested. Nine antibodies were, similar to the majority of idiopathic or drug-induced autoantibodies, not or only weakly reactive with Rh_{null} RBC. Three antibodies did not react with cord RBC and could be inhibited by soluble I antigen. The remaining four antibodies gave inhomogeneous reaction patterns or were even negative with selected RBC; their specificity could not be identified. On a Scatchard plot analysis of one ndab, a maximum of 173,000 drug-dependent antibodies of the IgG class can specifically bind per RBC in the presence of the drug. Although nomifensine and its metabolites do not attach tightly onto RBC, our results clearly indicate that RBC do not act as "innocent bystanders," but rather serve as a surface for (1) a loose attachment of drugs that possibly cause a subtle structural change in the cell antigens and, by this means, allow in vivo sensitization; and (2) a specific binding of the resultant antibodies. This concept would explain why these antibodies can be directed against drug-cell complexes, against cell antigens alone (autoantibodies), or against both in the same patient.

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NOMIFENSINE, an antidepressant drug, has been recently withdrawn because of the frequent occurrence of life-threatening immune-mediated intravascular hemolysis. In almost all cases this syndrome appeared to reflect the typical clinical and serological picture of the so-called immune complex-mediated immune hemolysis. The hemolysis was usually transitory, intravascular, and often associated with renal failure. In vitro studies have shown that the drug does not bind firmly to target cells and that the causative antibodies react with RBC only in the presence of the drug[1-4] and/or its metabolites, which leads to cell lysis by fixation of the terminal complement complexes.[5-7] The occurrence of autoimmune hemolytic anemia secondary to therapy with the drug has also been noted.[8,9]

A major, yet unresolved problem in this syndrome is the mechanism of sensitization and the true antigenic sites that determine the specificity of the resultant antibodies. The prevailing notion anticipates that drugs (haptens) first conjugate with some soluble noncellular macromolecule to give rise to the subsequent formation of high-affinity antibodies. These antibodies then combine with drugs to form antibody-drug complexes that may attach nonspecifically to RBC by an "innocent bystander" mechanism.[10] This hypothesis is based on the assumption that immunization does not require conjugation of the drug with RBC and that the antibodies produced do not have specificity for erythrocyte antigens.[10] We have recently identified in one patient with nomifensine-induced immune hemolysis multiple nomifensine (metabolites)-dependent antibodies (ndab) that recognize Rhesus blood group antigens.[11] In another report the presence of receptors on RBC for certain drugs or drug-antibody complexes has been suggested.[12] Therefore, we have reexamined a large number of ndab in order to see whether they exhibit specificity for known blood group antigens.

Our results show that 16 out of 30 antibodies do indeed have specificity that is not only controlled by the drug and/or its metabolites but also by defined RBC antigens, particularly of the Rhesus and I/i systems. These findings provide, we believe, supportive evidence that in some circumstances in vivo sensitization to drugs may be related to the formation of loose, but immunogenically effective drug-cell complexes.

MATERIALS AND METHODS

Antibodies. Serum samples of 30 patients with clinical and laboratory criteria consistent with the diagnosis of drug-induced immune hemolytic anemia (DIHA) of the so-called immune complex type were studied.[6,9] Each serum had ndab of the IgG and/or IgM classes invariably reactive with pooled normal RBC in the presence of ex vivo antigen[6,9] of the drug (urine containing the drug and its metabolites). Additionally, 22 eluates or sera containing RBC IgG autoantibodies (seven induced by nomifensine, four by α-methyldopa, one by cianidanol [(+)-2-(3,4-dihydroxyphenyl)-3,5,7-chromatriol], and ten unselected "idiopathic" autoantibodies) were concomitantly studied.

RBC. The following RBC were used: (1) pooled RBC freshly obtained from three or four healthy blood donors of group O with all major Rhesus factors, ie, D, C, c, E, e; (2) A_2 Rh_{null} RBC, kindly provided by Professor S. Seidl (Frankfurt, FRG); (3) two commercially available panels of O RBC (Ortho Pharmaceutical Corp, Raritan, NJ); (4) a panel of RBC lacking high-frequency antigens or having rare antigens (see the legend to Table 1) was kindly supplied by the Medical Research Council Blood Group Unit, London.

Drugs. The ex vivo antigen was urine of a volunteer collected 4.5 hours after the ingestion of 200 mg nomifensine. Purified nomifen-

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sine was kindly provided by Hoechst Pharmaceuticals (Frankfurt, FRG).

Serological procedures. The reactivity of antibodies was tested as described.[6,9] Serum samples were titrated against the cells in the presence of urine before (control) and after drug ingestion (ex vivo antigen). If the sera were incompatible with the cells to be tested due to isoagglutinins (anti-A, B) samples were preabsorbed with pooled A_1 or B RBC. After incubation for two hours at 37°C, the reactions were read visually for agglutination. Thereafter the RBC were washed three times in saline and tested by the indirect antiglobulin test using polyvalent antiglobulin serum (Ortho).

Inhibition tests. Drug-dependent antibodies that showed reactivity against adult but not against cord RBC were reinvestigated with adult RBC in the presence of defatted and preheated human milk (ten minutes in boiling water) as described for anti-I.[13]

Absolute number of antibodies attached per RBC. Plasma containing a pure nomifensine-dependent IgG antibody (no. 1, a gift from Dr B. Habibi, Paris) was incubated with 10×10^6 RBC (in 0.02 mL saline) in the presence of nomifensine or saline (control). In preceding experiments the amount of plasma and drug needed for maximum binding of antibodies to RBC had been determined (0.1 mL of plasma and 0.05 mL of a solution of 0.1 g/dL [wt/vol] nomifensine dissolved in 0.15 mol/L saline methanol [1:10]). After incubation for two hours at 37°C the RBC were separated by centrifugation through 1 mL of calcium-free Tyrode buffer (0.13 mol/L NaCl, 27 mmol/L KCl, 16 mmol/L $NaHCO_3$, 0.36 mmol/L $Na_2H_2PO_4$, 5 mmol/L $MgCl_2$-$6H_2O$, pH 7.2) that was supplemented with 0.2% bovine serum albumin, 1% glucose,[14] and 0.001 g/dL nomifensine (final concentration) to maintain maximal binding of the antibodies onto the cells.[6] The cells were resuspended in 0.1 mL of saline containing the drug, transferred to fresh tubes, and reincubated with serial dilutions of radiolabeled[15] and chromatographically purified polyclonal $F(ab)_2$ antihuman IgG (Fc) antibodies (Jackson Immunoresearch Laboratories, Avondale, PA). After incubation (30 minutes at room temperature) the cells were centrifuged again through the same buffer, and the radioactivity of the RBC pellet was measured. Centrifugation through this buffer completely eliminates unbound molecules.[14] All experiments were run in triplicate, including the controls.

RESULTS

All antibodies reacted with pooled O RBC in the presence of ex vivo antigen (agglutination and/or positive indirect antiglobulin test), but in no case in the presence of urine before ingestion of the drug (negative control). Fourteen antibodies reacted with Rh_{null} RBC as well as with other rare or common RBC whether they were tested undiluted or diluted. In contrast, 16 antibodies showed inhomogeneous reaction patterns with the RBC tested, and the majority of them had specificity to defined RBC antigens (Table 1). Nine of these antibodies were not or only weakly reactive with Rh_{null} cells, and three antibodies (nos. 5, 22, 35) were negative with cord RBC. Against a panel of common RBC, some of the former antibodies, if they were available in sufficient amounts, also exhibited specificity to "simple" Rh antigens, ie, C, c, f, or to the I antigen (Table 1).

The I specificity was also confirmed by inhibition tests using soluble I antigen in human milk (Table 2). Although the remaining four antibodies (nos. 4, 17, 18, 30) seemed to recognize certain RBC antigens, their specificity could not be identified.

To study the binding characteristics of the antibodies with homogeneous reaction patterns, we analyzed the attachment of a representative antibody by Scatchard plot analysis. For this experiment, a pure IgG ndab[1] was chosen. If it is assumed that one molecule of the $F(ab)_2$ binds to one molecule of human IgG, the maximum amount of antibody binding sites was 173,000 per RBC (Fig 1). This value is, however, only an approximation since the antibodies used

Table 1. ndab With Specificity for RBC Antigens

	Reciprocal Titer (Agglutination/AGT)		Differentiation With	
Case*	Pool	Rh_{null}	Commercial Panels	Rare Panel†
7	64/512	–/–	Strong only with c	Strong only with c
8	2/64	–/–	NT	NT
11	2/8	–/1(w)	NT	NT
14	–/16	–/–	IHRP	IHRP
20	64/16	1/1	Strong only with C	Strong only with C
23	1/16	–/1(w)	NT	NT
27	1/32	1/1(w)	Strong only with f	IHRP
41	1/4	–/–	NT	NT
49	–/8	–/–	IHRP	IHRP
5	–/4	–/4	Negative with cord RBC	NT
22	–/8	–/8	Negative with cord RBC	NT
35	4/16	4/16	Negative with cord RBC	IHRP
4	8/1(w)	4/1(w)	IHRP	IHRP
17	8/1(w)	8/1(w)	IHRP	Negative with cD(e)‡
18	16/2	16/2	IHRP	Negative with pp RBC
30	–/4	–/2	IHRP	NT

Abbreviations: AGT, antiglobulin test; IHRP, inhomogeneous reaction pattern; NT, not tested; (w), weak reaction.

*Indicates the number of patients in previous studies.[6,9] The results with normal RBC show some discrepancies with our previous studies since another sample of ex vivo antigen and often different serum samples of individual patients had to be used.

†The following antigens were considered: Ko, Kn(a–)McC(a–), Lan–, LKE–, U–Fy(a–b–), Yt(a–), –D–/–D–, O_h, H–, Co(a–), pp, cD(e), Co(a–), hr^{B-}, Jk(a–b–), Lu(a–b–), Ge:–2,3, Rg(–)Cs(a–), Vel–, P_2^k, and Lw(a–b–).

‡Detailed information on this cell has been previously reported.[16]

Table 2. Identification of Anti-I Specificity in Three Cases With Nomifensine-Dependent Antibodies

Case	Reciprocal Titer (Agglutination/AGT)				
	Adult O Rh R_1R_2		Cord RBC		
	Saline	Human Milk	I	II	II
22	–/8	–/2	–/–	–/–	–/–
5	–/4	–/1	–/–	–/–	–/–
35	4/16	1/4	–/–	–/–	–/–
21*	64/4	128/8	64/4	64/4	64/4
Anti-I†	32/NT	8/NT	4/NT	4/NT	4/NT

*Control (nomifensine-dependent antibody of those giving homogeneous reactions with all RBC tested).

†Diluted 1:20.

were polyclonal and only one ndab was studied. The value(s) would be more precise if purified and radiolabeled $F(ab)_2$ fragments of ndab were used. Nevertheless, the straight-line extrapolation in Fig 1 would suggest that the antibody studied was of restricted heterogeneity. When the serum was incubated with the same cells in the absence of the drug (control), no binding of antibody was seen. Washing of the antibody-coated cells in the absence of drug removed at least 70% of cell-bound antibodies.

In comparison with ndab, ten unselected idiopathic and 12 drug-induced autoantibodies were tested against the same RBC. In all groups approximately 50% of the antibodies were not reactive with Rh_{null} cells (Table 3), thus indicating the absence of identical binding sites for all these antibodies.

DISCUSSION

Since the pioneer work of Landsteiner,[17] it has generally been accepted that haptens, usually substances of a molecular weight of less than 1,000 daltons, can acquire immunogenicity only after coupling to a macromolecular carrier. This compound antigen, ie, hapten-protein complex, will then display a multitude of antigenic sites that may provoke the production of various antibody populations with different specificities.[18-20]

Table 3. Reactivity of Idiopathic and Drug-Induced Autoantibodies Against Normal and Rh_{null} RBC

Autoantibodies (Total)	Induced by (Drug)	Not Reactive With Rh_{null}
7*	Nomifensine	4
4	α-Methyldopa	3
1†	Cianidanol	1
10	Idiopathic	5

All autoantibodies were eluates from autologous or homologous RBC.

*The autoantibodies in patients 48 and 50[9] are included. Both of them were, like the drug-dependent antibodies in the same patients, reactive with Rh_{null} cells.

†Not yet published.

Immune-mediated cytopenias are well-known hazards of drug therapy. However, the etiology of this disorder and the molecular basis of drug-antibody-cell interactions have remained largely speculative. Ackroyd[21] postulated that the drug acts as a hapten and the resultant antibodies bind to drug-coated cells, which leads to complement activation on the cell surface. This concept was called in question by the fact that most drugs involved in drug-induced cytopenias do not bind firmly to target cells.[10] An alternative model claimed that drugs form stable compounds with plasma macromolecules that react with antibody to form immune complexes that then attach nonspecifically onto the target cells.[22,23] But neither the mechanism proposed by Ackroyd nor the hypothesis of nonspecific attachment of drug-antibody complexes can satisfactorily explain the occurrence of drug-induced autoimmune hemolytic anemias, ie, by α-methyldopa.[24]

Of the three possible mechanisms of DIHA the so-called immune complex type is clinically and serologically by far the least understood form.[10] In our recent reports on a total of 51 patients with nomifensine-induced immune hemolysis,[5,6,9,11] the majority of patients (42 cases) appeared to belong in the category of the immune complex type as judged from clinical as well as serological findings, whereas five

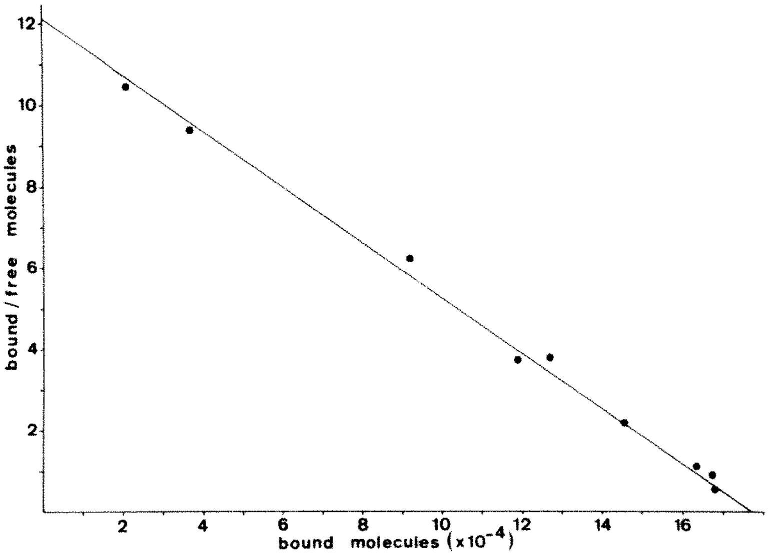

Fig 1. Absolute number of anti-IgG molecules bound per RBC preincubated with nomifensine-dependent antibody in the presence of the drug. The background radioactivity of RBC preincubated with the same serum in the absence of the drug was measured concomitantly (control). This background radioactivity (<0.01%) was subtracted from the total amount of cell-associated radioactivity for each dilution of radiolabeled antibody.

patients had developed a true autoimmune hemolytic anemia and three patients had both types of immune-mediated hemolysis. Thus, the most convincing way to address the question whether or not the RBC act as a carrier surface for the drug and its metabolites would be to demonstrate specificity of drug-dependent antibodies for defined RBC antigens. This prompted us to reinvestigate all nomifensine-induced RBC antibodies that were available in our laboratory. In fact, most antibodies studied required certain RBC antigens for full reactivity. This indicated that the target cells in this syndrome must supply a part of (in the case of drug-dependent antibodies) or even the entire area (in the case of drug-induced autoantibodies) for sensitization and for the subsequent attachment of the resultant antibodies. This is corroborated by several lines of evidence. First, the drug-dependent antibodies recognize only one cell type, ie, RBC and not platelets. Second, binding characteristics onto the cells exactly resemble that of true RBC antibodies in that absorbed and eluted drug-dependent antibodies can be shown to rebind by their F(ab) rather than by their Fc portion.[6] Third, the results of the Scatchard plot analysis in this study speak for specific rather than for unspecific attachment of the antibodies to the cells. In our experience, unspecific attachment of IgG antibodies to RBC cause usually weak reactions in the antiglobulin test and in radioimmunoassays. Fourth, the heterogeneity of the ndab among patients or even in the same patient (drug-dependent antibodies and/or autoantibodies) corresponds to that seen in animals sensitized with hapten-protein conjugates. Finally, some workers have, in isolated cases, reported specificity of drug-dependent antibodies.[12,25,26]

Thus we have reason to assume that loose binding of drugs (haptens) on cell surfaces is sufficiently immunogenic for sensitization against cell-drug complexes and/or against the cells alone. The failure to recognize in vitro the causative drugs on target cells does not preclude an interaction with blood cells in vivo. Continued provision of drug into the circulation might maintain the unbound drug concentration at a level favoring such association, whereas a less favorable gradient develops in an in vitro test carried out at a single drug concentration. If this is true, it is likely that a prolonged loose attachment of drugs and/or their metabolites onto the cells can alter natural antigens in such a way that allows the production of heterogeneous antibodies.

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Cytoplasmic Immunoglobulins in Chronic Lymphocytic Leukemia B Cells

By G. Pianezze, I. Gentilini, M. Casini, P. Fabris, and P. Coser

It is generally assumed that chronic lymphocytic leukemia of B cell origin (B-CLL) is characterized by the presence of surface membrane immunoglobulins (SmIg) and by the absence of cytoplasmic immunoglobulins (CyIg). In a variable number of cases SmIg are not detectable because of their low density on the cellular surface. Because a constant presence of CyIg in 20 subjects suffering from B-CLL has been reported recently, we reexamined 15 SmIg-negative and 10 SmIg-positive B-CLL patients by SmIg and CyIg determinations. We used a direct immunofluorescence method on peripheral blood mononuclear cells for the detection of SmIg and, after fixation, for CyIg. CyIg were detectable in 24 out of 25 cases, with a fluorescence intensity ranging from weak to moderate. The existence of frequent negative results for CyIg determination in B-CLL reported in the literature probably depends on the low sensitivity of the method used. We conclude that CyIg determination is useful in phenotyping every B-CLL patient, especially SmIg-negative ones.

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CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) is a malignant proliferation of monoclonal lymphocytes of B cell origin in most instances.[1,2] It is generally accepted that B-CLL cells are characterized by the presence of surface membrane immunoglobulins (SmIg), the absence of cytoplasmic immunoglobulins (CyIg), the presence of mouse red cell receptors, and a predominance of receptors for $C3_d$ over those for $C3_b$.[3,4]

It is also common knowledge that, in the majority of SmIg-positive B-CLL, the intensity of surface immunofluorescent staining varies from weak to moderate,[5] and if quantitatively measured, the amount of SmIg appears to range from a quarter to a tenth when compared with that of normal lymphocytes.[6] A variable percentage (0% to 30%) of SmIg-negative B-CLL has been described by various authors.[4,7-9] To explain this finding, it is generally assumed that the density of SmIg on B cells in such cases is so weak that it is not detectable by common immunofluorescence methods. In such cases it is not possible to demonstrate the monoclonality of the B lymphocyte population supposed leukemic.

In recent publications[10,11] the presence of CyIg in B-CLL cells is described. In one of them the authors affirm that CyIg determination is superior to SmIg determination to prove the monoclonality of leukemic cells.[10] This statement seems to be in contrast with the current opinion; for that reason we decided to check it in our laboratory by reexamining a group of patients affected by B-CLL.

MATERIALS AND METHODS

Patient population. We studied 25 patients previously diagnosed as having B-CLL. This group included all patients in whom the presence of SmIg could not be demonstrated (15 cases) and others chosen at random among those SmIg-positive (ten cases). At diagnosis all patients showed a lymphocyte count above 10×10^9/L in peripheral blood (PB). Diagnosis had been made on the basis of typical clinical and laboratory findings. All patients were staged with the classification of Rai and colleagues.[12] Table 1 shows the principal clinical and laboratory findings of the patient population at diagnosis.

Surface markers. The mononuclear cells from B-CLL patients were separated from PB by a density gradient (Ficoll-Hypaque).[13] The presence of SmIg on mononuclear cells was determined through a direct immunofluorescence method[14] using fluorescein-conjugated antisera specific to human light and heavy chains (Dako Corp, Santa Barbara, CA). Fluorescence was always examined using both a fluorescent microscope (Ortholux with a xenon lamp, 75 W; Leitz, Wetzlar, Germany) and a laser cytofluorograph (Spectrum III:

Table 1. Stage of Patients and Laboratory Findings of Their Lymphocytes, Evaluated at Diagnosis

Case	Stage*	Lymphocytes ($\times 10^9$/L)	SmIg Phenotype	Surface Markers	
				DR (%)	OKT1 (%)
1	0	29.2	δ μ λ	78	30
2	0	20.8	/	90	25
3	0	21.1	μ κ	79	45
4	1	30.7	/	94	84
5	1	13.6	δ γ λ	89	84
6	0	10.5	/	74	30
7	1	15.1	/	95	42
8	4	53.2	/	95	85
9	0	11.0	/	78	25
10	1	18.9	μ λ	96	75
11	0	32.4	/	87	32
12	0	25.9	/	88	41
13	0	12.0	/	67	67
14	0	24.3	/	91	15
15	0	27.4	δ κ	89	15
16	0	24.6	/	94	57
17	0	11.2	κ	79	84
18	1	10.3	μ κ	81	90
19†	2	39.0	μ λ	92	22
20	2	13.9	γ λ	75	83
21	1	40.9	/	87	94
22	1	54.0	/	71	83
23	2	51.2	/	96	15
24	0	55.1	λ	93	84
25	2	39.9	/	91	92

*The stage was evaluated according to the classification of Rai and colleagues.[12]

†This patient showed a serum M component (IgM-λ).

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Ortho Diagnostics, Westwood, MA). The mononuclear cells were tested with the monoclonal antibodies OKDR, OKT1 (Ortho Pharmaceuticals, Raritan, NJ), and Leu 1 (Becton Dickinson, Mountain View, CA) with the direct and indirect immunofluorescence methods recommended by Ortho Diagnostics for their cytofluorograph.

CyIg. Mononuclear cells were washed three times with phosphate-buffered solution (PBS) and smeared on slides by cytocentrifugation. The slides were subsequently fixed for 20 minutes in absolute methanol at 4°C and air-dried. The cell smear was then covered with chain-specific fluorescein-conjugated antisera (Dako) optimally diluted (1:40 was, in our experience, the optimal dilution). After 30 minutes' incubation at room temperature the slides were washed three times in PBS on a mechanical agitator (five minutes for each washing); at the last washing an Evans' blue solution (one drop every 100 mL PBS) was added as a counterstain. The slides were mounted in glycerol-PBS and observed under a fluorescent microscope.

To exclude the possibility that the fluorescence observed with the method for CyIg determination was due to SmIg, we used a technical procedure called neutralization. The suspension of mononuclear cells was previously incubated with an antihuman immunoglobulin antiserum (without fluorescein) for 30 minutes, then cytocentrifuged on microscope slides, fixed, and incubated for another 30 minutes with chain-specific fluorescein-conjugated antisera. That way the SmIg were neutralized by the unmarked antibody, and after fixation, the marked antibody could detect only the CyIg. We used T-CLL cells as negative controls for both SmIg and CyIg.

RESULTS

Data concerning SmIg are compared with those related to CyIg in Table 2, which shows that CyIg were detectable in 24 out of 25 cases (in three of them we could detect μ-type heavy chains but no light chains). In all cases, the fluorescence intensity for CyIg in the majority of cells, even if weaker than that expressed by more differentiated subtypes of the B cell line (plasma cells and plasmacytoid lymphocytes), allowed a clear distinction between positive and negative cells (Fig 1). Only a fraction of cells variable from 1% to 3% showed a strong fluorescence intensity.

Keeping in mind the difficulty in locating cell structures through conventional optical microscopy and because the effect of fixation on SmIg distribution is not completely known, we have considered the possibility that the positiveness detectable with our CyIg method might be attributable to SmIg, even if their fluorescence distribution patterns are always completely different. SmIg neutralization with unmarked antibodies excluded such a possibility; as a matter of fact, after neutralization SmIg were no longer detectable, whereas CyIg did not show any reduction in fluorescence intensity (Fig 2).

DISCUSSION

The results of our investigation substantially agree with the data published by Han et al[10] who found monoclonal CyIg in all 20 cases of B-CLL examined. In the same article they reported that in each B-CLL patient the intensity of SmIg fluorescence was moderate to strong. In some cases it was not easy to demonstrate the monoclonality of the leukemic population, probably because of nonspecific

Table 2. SmIg and CyIg Evaluation on the Lymphocytes of the Patients Studied

	SmIg			CyIg		
Case	Fluorescence Intensity	Positive Cells (%)	Phenotype	Fluorescence Intensity	Positive Cells (%)	Phenotype
1	+ + +	75	δ μ λ	+ − −	70	δ μ λ
2	−	/	/	+ − −	90	δ κ
3	+ − −	30	μ κ	+ − −	50	μ κ
4	−	/	/	+ − −	40	μ λ
5	+ − −	40	δ γ λ	+ − −	70	μ λ
6	−	/	/	+ − −	30	μ
7	−	/	/	+ − −	80	μ
8	−	/	/	+ − −	60	μ κ
9	−	/	/	+ − −	70	μ κ
10	+ − −	30	μ λ	+ − −	60	μ λ
11	−	/	/	+ − −	85	μ
12	−	/	/	+ − −	75	μ λ
13	−	/	/	−	/	/
14	−	/	/	+ − −	65	μ κ
15	+ − −	30	δ κ	+ − −	80	μ κ
16	−	/	/	+ − −	70	μ λ
17	+ − −	30	κ	+ + −	65	μ κ
18	+ − −	40	μ κ	+ − −	80	μ κ
19*	+ + +	90	μ λ	+ + −	90	μ λ
20	+ + −	65	γ λ	+ − −	70	γ λ
21	−	/	/	+ − −	75	μ κ
22	−	/	/	+ + −	75	μ κ
23	−	/	/	+ − −	55	γ κ
24	+ − −	35	λ	+ − −	90	μ λ
25	−	/	/	+ + −	70	μ λ

Key: −, negative result; + − −, weak positiveness; + + −, moderate positiveness; + + +, strong positiveness.

*This patient showed a serum M component.

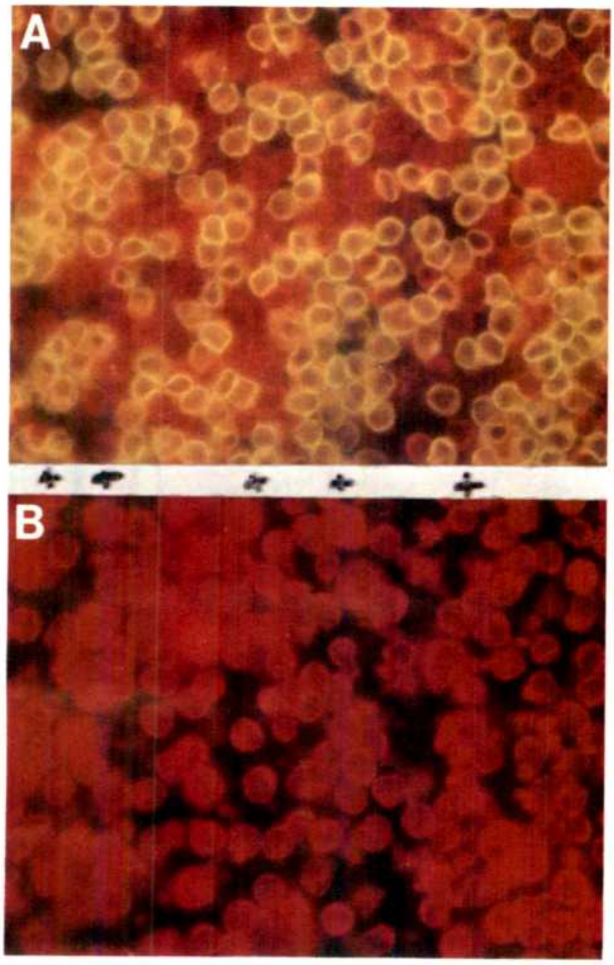

Fig 1. The photomicrograph (original magnification × 400) shows the results of CyIg immunofluorescence staining in case 9. (A) Positive staining with an anti-κ antiserum. (B) Negative staining with an anti-λ antiserum.

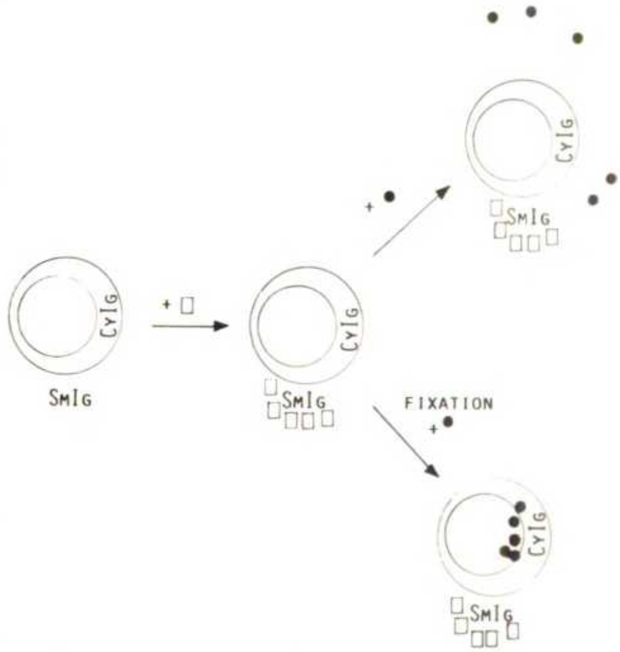

Fig 2. The non-fluorescein-conjugated antihuman immunoglobulin antiserum (□) reacts and neutralizes the SmIg. Under such conditions an FITC-conjugated antihuman immunoglobulin antiserum (●) cannot detect either the SmIg or the CyIg. Immunofluorescent staining will be negative, thus confirming the SmIg neutralization. After fixation, an FITC-conjugated antihuman immunoglobulin antiserum (●) is able to penetrate into the cell and detect the CyIg. Immunofluorescent staining will therefore be positive.

absorption of serum immunoglobulins by Fc receptors. They affirm that CyIg determination is superior to SmIg determination in B-CLL cell phenotyping because fixation suppresses the activity of Fc receptors.

On the other hand, we noticed the usefulness of CyIg determination in B-CLL cells, particularly when SmIg detection was not possible as a consequence of their low density on the cellular surface. Also, Schroff et al[11] reported the presence of monoclonal CyIg in all three cases examined of SmIg-negative B-CLL. Koziner et al[4] reported constant negative results for CyIg determination in 60 cases of B-CLL, whereas Gourdin et al[15] demonstrated, by immunoelectron microscopy, monoclonal CyIg only in B-CLL with serum M component. Furthermore Rudders[16] had reported the presence of monoclonal CyIg in 11 cases of B-CLL out of 26, and only four of them had serum M component.

To explain these conflicting results among the different reported findings, we should admit either the existence of B-CLL with a different CyIg phenotype (positive or negative) or a different sensitivity of the methods used. The second hypothesis seems to be more acceptable since a spatiotemporal clustering of so many B-CLL with the same CyIg phenotype appears statistically unlikely.

There is increasing evidence demonstrating that cells from B-CLL patients secrete monoclonal immunoglobulins. Using more sensitive methods than traditional ones, their presence may be demonstrated in the serum and/or urine of such patients in a percentage of cases ranging from 53% to 73%, depending on the method used.[17,18] These findings suggest an intrinsic but poorly developed secretory capacity in B-CLL cells and therefore indirectly justify the presence of monoclonal CyIg in the leukemic cells.

Gourdin et al[16] considered that, excluding cases with a high M component, the presence of monoclonal immunoglobulins in serum of typical B-CLL depends on a small leukemic subpopulation that is also positive for CyIg. On the other hand, on the basis of our results and of those of Han et al,[10] this property seems to be a characteristic of a wide majority of the leukemic population. In conclusion, even if we recognize the necessity of finding an adequate explanation for the reported discrepancies among data in the literature, in our hands the CyIg determination is a valid help in improving the sensitivity and specificity of B-CLL phenotyping.

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Total Body Irradiation and High-Dose Etoposide: A New Preparatory Regimen for Bone Marrow Transplantation in Patients With Advanced Hematologic Malignancies

By K.G. Blume, S.J. Forman, M.R. O'Donnell, J.H. Doroshow, R.A. Krance, A.P. Nademanee, D.S. Snyder, G.M. Schmidt, J.L. Fahey, G.E. Metter, L.R. Hill, D.O. Findley, and I.J. Sniecinski

In a phase I/II study, 47 patients (median age, 24 years) with hematologic malignancies (33 patients with acute leukemia not in first remission and 14 patients with other advanced malignant hematologic disorders) were treated with total body irradiation and high doses of etoposide (VP16-213) followed by bone marrow transplantation. At the time of analysis, 21 patients were alive, and 19 of them were in continued complete remission for 101 days to >40 months (median, 12 months). The actuarial disease-free survival rate of the 33 acute leukemia patients is 43% (2 SEM, 18%) and the actuarial relapse rate is 32% (2 SEM, 20%). Five of the 14 patients with the other hematologic malignancies are alive, and four of them continue to be free of disease for 8 to 27 months. Pharmacokinetic studies established a strong correlation between the administered drug doses and their plasma levels and also demonstrated complete drug clearance prior to marrow grafting. An etoposide dose of 60 mg/kg body weight was found to be the maximum tolerated dose. This new preparatory regimen was well tolerated and was not associated with specific acute or long-term regimen-related toxicities. Our data suggest that total body irradiation with high-dose etoposide presents a viable alternative to other preparatory regimens. The role of this novel combination remains to be defined by future prospective randomized trials.

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RECURRENCE of the underlying malignancy after bone marrow transplantation (BMT) is the leading cause for failure in patients who are not in first complete remission (CR) of acute leukemia or no longer in the chronic phase of chronic myelocytic leukemia (CML) when preparation for BMT is begun. Disease-free survival rates for such candidates after myeloablation and immunosuppression with total-body irradiation (TBI) and high-dose cyclophosphamide followed by BMT range from 10% to 40%.[1-9] With the aim of reducing the relapse rate without increasing regimen-related toxicities, we have developed a preparatory regimen consisting of TBI and high doses of the epipodophyllotoxin-derivative etoposide (VP16-213). Etoposide is almost exclusively marrow toxic and has proven activity against malignant cells if administered in standard doses to patients with relapsed hematologic malignancies[10-16] or in high doses followed by autologous bone marrow rescue.[17] Another advantage is its ability to penetrate into the CSF after high-dose intravenous administration.[18] The new combination, TBI with etoposide, was tested in a phase I/II study on 33 patients with acute leukemia who either had never attained CR or had relapsed at least once; in addition, 14 patients with other poor-risk hematologic malignancies were entered onto this study.

PATIENTS AND METHODS

Patients. Between March 1983 and April 1986, 47 patients (median age, 24 years; range, 1 to 47 years) with hematologic malignancies were prepared for BMT with TBI and high-dose etoposide. Twenty patients had acute nonlymphoblastic leukemia (ANLL): fifteen either had failed to ever attain CR with chemotherapy or had relapsed after successful remission induction; five were in second CR (Table 1). The subtyping of ANLL was performed according to French-American-British (FAB) criteria.[19] Thirteen patients had acute lymphoblastic leukemia (ALL): eight either had not entered a first CR in spite of repeated remission induction attempts or were in relapse; five patients were in second or third CR (Table 2). Patients identified as "induction failure" had received at least two courses of combination chemotherapy without attaining CR. The ten patients with acute leukemia who were in second or third CR at the time of BMT previously had first remissions that lasted 2 to 36 months (median, 7 months), and all had relapsed while receiving maintenance chemotherapy. In addition, 14 patients with other hematologic malignancies (Table 3) were enrolled in this study: two had refractory anemia with excess blasts (RAEB), two had refractory anemia with excess blasts in transition (RAEBIT), five had CML in accelerated or blastic phase, two had chronic myelomonocytic leukemia (CMMoL), two had Hodgkin's disease (HD) refractory to radiation and repeated courses of combination chemotherapy, and one patient had myelofibrosis/sclerosis (MF/S). Patients with myelodysplastic syndromes were classified according to a previously published system.[20]

Preparatory regimen. To 39 patients, TBI was administered in 11 fractions of 120 cGy between day −7 and day −4 to a total dose of 1,320 cGy.[21] An electron boost of 400 cGy was given to the testes on day −7 and one of 300 cGy twice daily to the chest on days −5 and −4. Because of limited availability of the linear accelerator, the remaining eight patients received a single-dose TBI regimen (750 cGy at 26 cGy/min) on day −5.[22] Etoposide was given by a four-hour infusion (1 mg etoposide/mL saline) on day −3 (Fig 1). Because of the instability of this drug, infusion bottles were changed every hour during the four-hour infusion period. Vigorous hydration

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Table 1. Pretransplant Characteristics, TBI Method, Etoposide Dose, and Outcome of BMT for 20 Patients With ANLL

UPN	Age (yr)	Disease Type	Disease Status	Blasts in Bone Marrow	TBI	Etoposide (mg/kg)	Treatment Result	KS
183	29	ANLL-M2	IF	35%	S	25	Alive in CCR on day +1242	100
192	30	ANLL-M1	1 RL	85%	F	30	Relapsed day +160, died day +418	0
219	19	ANLL-M1	IF	35%	F	30	Died on day +147 with IP	0
221	20	ANLL-M2	IF	57%	F	30	Alive in CCR on day +934	70
222	29	ANLL-M4	IF	13%	F	30	Alive in CCR on day +927	100
223	1	ANLL-M4	IF + EL	25%	S	40	Relapsed day +100, died day +147	0
234	14	ANLL-M2	2 RL	9%	F	40	Alive in CCR on day +837	90
237	28	ANLL-M2	4 RL + EL	28%	F	40	Relapsed day +313, died day +493	0
245	25	ANLL-M4	1 RL	35%	S	50	Alive in CCR on day +774	90
272	33	ANLL-M2	1 RL	81%	F	60	Died on day +143 with IP	0
281	8	ANLL-M2	2 RL	96%	S	60	Relapsed day +83, died day +127	0
287	43	ANLL-M2	1 RL + EL	45%	F	70	Died on day +47 with GVHD & IP	0
292	17	ANLL-M4	2 RL + EL	8%	F	70	Died on day +52 with sepsis & IP	0
295	28	ANLL-M4	1 RL	95%	F	70	Died on day +56 with IP	0
305	12	ANLL-M2	IF	68%	F	60	Relapsed day +139, died day +296	0
236	29	ANLL-M2	2 CR	<5%	F	40	Alive in CCR on day +822	100
251	6	ANLL-M1	2 CR	<5%	F	40	Relapsed day +90, died day +217	0
257	36	ANLL-M2	2 CR	<5%	F	50	Alive in CCR on day +679	100
270	12	ANLL-M3	2 CR	<5%	F	60	Died on day +73 with graft failure	0
314	37	ANLL-M2	2 CR	<5%	F	60	Died on day +53 with GVHD & IP	0

Abbreviations: KS, Karnofsky score; IF, Induction failure; RL, relapse; EL, extramedullary leukemia; S, single-dose TBI; F, fractionated TBI; CCR, continued CR; IP, interstitial pneumonia.

Table 2. Pretransplant Characteristics, TBI Method, Etoposide Dose, and Outcome of BMT for 13 Patients With ALL

UPN	Age (yr)	Disease Type	Disease Status	Blasts in Bone Marrow	TBI	Etoposide (mg/kg)	Treatment Result	KS
302	9	ALL-CALLA	2 RL	10%	F	60	Relapsed on day +387	100
304	36	ALL-Ph^1+	IF	8%	F	60	Died on day +121 with GVHD & IP	0
306	25	ALL-Ph^1+	1 RL	14%	F	60	Alive in CCR on day +375	100
309	23	ALL-Ph^1+	IF	20%	S	60	Died on day +93 with GVHD & IP	0
315	18	ALL-Null	3 RL + EL	9%	F	60	Alive in CCR on day +326	100
324	6	ALL-CALLA	2 RL	38%	F	60	Alive in CCR on day +269	100
343	22	ALL-Null	1 RL	57%	F	60	Died on day +51 with GVHD & IP	0
355	23	ALL-Ph^1+	1 RL	23%	F	60	Alive in CCR on day +101	100
311	18	ALL-CALLA	2 CR	<5%	F	60	Alive in CCR on day +347	100
330	6	ALL-CALLA	2 CR	<5%	F	60	Alive in CCR on day +242	100
331	7	ALL-CALLA	3 CR + EL	<5%	F	60	Died on day +55 with IP	0
348	11	ALL-Null	2 CR	<5%	F	60	Alive in relapse on day +135	60
351	8	ALL-CALLA	2 CR	<5%	F	60	Alive in CCR on day +117	100

Abbreviations: CALLA, common acute lymphocytic leukemia; Ph^1+, Philadelphia chromosome–positive.

Table 3. Pretransplant Characteristics, TBI Method, Etoposide Dose, and Outcome for 14 Patients With Hematologic Malignancies Other Than Acute Leukemia

UPN	Age (yr)	Disease Type	TBI	Etoposide (mg/kg)	Treatment Result	KS
228	19	RAEB	F	40	Relapsed day +462, died day +653	0
233	41	CMMoL	F	40	Alive in CCR on day +833	100
256	39	RAEB	F	50	Alive in CCR on day +704	100
263	34	RAEBIT	F	50	Died on day +72 with GVHD & IP	0
266	40	MF/S	F	50	Alive in CCR on day +654	100
267	28	HD	F	60	Alive in relapse on day +644	70
277	24	HD	F	60	Relapsed day +343, died day +432	0
288	29	CML-BP	S	70	Relapsed day +131, died day +183	0
291	4	RAEBIT	S	70	Relapsed day +30, died day +88	0
299	47	CML-AP	F	70	Died on day +205 with GVHD & IP	0
301	24	CML-BP	S	70	Relapsed day +93, died day +127	0
312	43	CML-AP	F	60	Relapsed day +60, died on day +256	0
329	26	CML-BP	F	60	Alive in CCR on day +249	100
333	41	CMMoL	F	60	Died on day +66 with GVHD & IP	0

Abbreviations: CML-BP, CML in blastic phase; CML-AP, CML in accelerated phase.

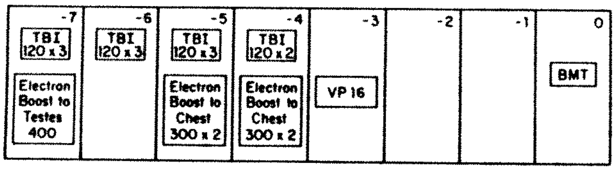
-7
-6
-5
-4
-3
-2
-1
0
TBI 120 x 3
TBI 120 x 3
TBI 120 x 3
TBI 120 x 2
Electron Boost to Testes 400
Electron Boost to Chest 300 x 2
Electron Boost to Chest 300 x 2
VP 16
BMT

Fig 1. Preparatory regimen consisting of TBI delivered between days −7 and −4 and infusion of high-dose etoposide (VP16) on day −3 followed by BMT on day 0.

(150 mL/m^2/h) and diuresis were performed for 72 hours after it was observed that etoposide could lead to hemorrhagic cystitis. Following a dose escalation schema, five patients were treated at level I (25 to 30 mg/kg), seven at level II (40 mg/kg), five at level III (50 mg/kg), seven at level IV (60 mg/kg), and seven at level V (70 mg/kg). After level IV was established as the maximum tolerated dose, another 16 patients received etoposide at 60 mg/kg. During and after drug administration, blood specimens were collected and analyzed for etoposide plasma levels by using high-performance liquid chromatography.[23]

Marrow transplantation. For 46 patients their respective histocompatible siblings and in one case (unique patient no. 228) an identical twin served as bone marrow donors. On day 0, the day of BMT, marrow was harvested from the donors, filtered for removal of particles, and administered intravenously to the recipients.[24] In the case of four recipients who had a major blood group mismatch with their donors, the red cells were removed from the marrow aspirates by centrifugation in a cell separator.[25]

Post-BMT care. Supportive care after BMT was provided as described previously[26] except that the patients on this study did not receive prophylactic granulolcyte support. Nonabsorbable antibiotics were given for gastrointestinal decontamination to all patients on this study.[27] Posttransplant immunosuppression for prevention of graft-*v*-host disease (GVHD) consisted of methotrexate/prednisone in 16 patients and of cyclosporine A/prednisone in 30 patients. Twelve patients who received methotrexate/prednisone and nine patients taking cyclosporine A/prednisone participated (along with 86 other leukemia patients) in a prospective randomized trial comparing these two drug combinations.[28] GVHD was graded according to previously described criteria.[29]

Grading of toxicity and performance status. The acute and subacute toxicity encountered during this study was graded according to generally accepted criteria.[30] The performance status was scored following the Karnofsky system.[31]

Statistical evaluation. The analysis of actuarial survival in continued CR and of probability for relapse were done by nonparametric estimation from incomplete observations.[32]

Approval. All clinical research protocols involved in this study had been approved by the Institutional Review Board of the City of Hope National Medical Center. Patient entry to study was contingent upon prior written informed consent.

RESULTS

Pharmacokinetic studies. Plasma etoposide levels rose steeply during the time of intravenous drug administration, followed by an equally rapid clearance ($t\frac{1}{2} = 7.5$ hours). Approximately 50 hours after etoposide infusion no appreciable amounts of the drug could be detected (Fig 2). After another 24 hours, the marrow graft was infused. The peak etoposide plasma levels ($r = .97$, $n = 15$) and the areas under the curves ($r = .74$, $n = 14$) of the patients in whom pharmacokinetic studies were carried out correlated well to the amount of drug administered. The complete pharmacokinetic data including drug excretion results will be presented elsewhere (J.H. Doroshow et al, in preparation).

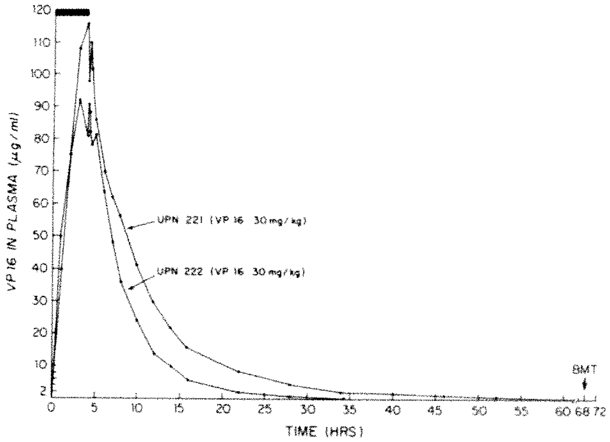

Fig 2. Etoposide (VP16) plasma levels (in μg/mL) of two patients (UPN 221 and 222) who received 30 mg etoposide/kg body weight by infusion over four hours (black horizontal bar). BMT was performed 68 to 72 hours after the beginning of drug administration.

Toxicity data. A transient, sometimes painful, rash (grade 2), mostly localized to the palms, soles, and periorbital area occurred at higher dose levels and lasted from four days to 3 weeks. Nausea and vomiting (grade 1 to 2) lasting for several hours occurred in patients who had received either 60 or 70 mg etoposide/kg body weight. Hypotension (grade 1) was encountered in one patient, presumably due to too vigorous diuresis with furosemide. No cases of arrhythmia were observed. Four patients developed hemorrhagic cystitis (grade 2), which was avoided in subsequent patients by maintaining a high urine flow for three days after etoposide infusion. We did not encounter any episodes of clinically significant metabolic acidosis. During the time of marrow aplasia, 41 patients developed clinical sepsis, ie, temperature >38.5°C in the absence of neutrophils. These 41 patients were treated with systemic broad-spectrum antibiotics. Severe stomatitis (grade 3 to 4) necessitating intermitten intravenous administration of narcotics occurred in most patients receiving the two highest drug doses. Stomatitis resolved in all patients at the end of the aplastic phase, ie, after 2 to 4 weeks. Two of the seven patients treated with 70 mg etoposide/kg developed severe jaundice and liver failure (grade 4) without signs of GVHD or venoocclusive disease. No neurotoxicity was observed in any of the 47 patients entered onto this study. The acute toxicity data are summarized in Table 4. No regimen-specific long-term toxicity was encountered. Two patients (UPN 221 and 245) have developed a clinical picture compatible with the chronic obstructive lung disease that is sometimes seen in recipients of allogeneic marrow grafts.

Engraftment. With the exceptions of one patient who had only transient signs of engraftment (UPN 270) and one patient with persisting malignancy (UPN 291) all BMT recipients showed a rise of WBC counts to >1,000/μL within 11 to 23 days (median, 14 days). Genetic marker analysis

Table 4. Toxicity Data Obtained in the TBI/VP16 Dose Escalation Study

	VP16 Dose (mg/kg)				
Symptom	25-30 (n = 5)	40 (n = 7)	50 (n = 5)	60 (n = 23)	70 (n = 7)
Rash	1	2	0	5	3
Severe nausea/emesis	1	0	0	4	3
Hypotension	0	0	1	0	0
Arrhythmia	0	0	0	0	0
Hemorrrhagic cystitis	1	1	1	1	0
Clinical sepsis*	5	6	4	19	7
Severe stomatitis	0	1	0	13	6
Hepatotoxicity	0	0	0	0	2
Neurotoxicity	0	0	0	0	0

*For a definition of clinical sepsis, see the text.

demonstrated hematopoiesis to be entirely of donor origin in all cases except for the two aforementioned patients with graft failure (UPN 270) or with persisting malignancy (UPN 291). Three patients (UPN 222, 233, and 234) have become stable hemopoietic chimeras as reported elsewhere.[33]

Survival results. At the date of evaluation (August 1, 1986), 16 of the 33 patients with acute leukemia were alive, and 15 were in continued CR. The minimum follow-up of patients surviving in continued CR was 101 days, and the maximum was 40 months (median, 12 months). As shown in Fig 3, the actuarial disease-free survival rate for acute leukemia patients was 43% (2 SEM, 18%). Five of the 14 patients with hematologic malignancies other than acute leukemia were alive, and four of them were in continued CR for 8 to 27 months.

Dose-effect relationship. The relationship between the doses of etoposide administered to the patients in the various groups (dose levels I to V) and the incidence of relapse and survival in these groups is summarized in Table 5.

GVHD. The following incidence of acute GVHD was observed: grade 0 in 17 patients, grade I in ten patients, grade II in ten patients, grade III in seven patients, and grade IV in two patients; ie, the incidence of no or mild acute GVHD (grade 0 or I) was 59% and of moderate-to-severe acute GVHD (grade II to IV) 41%. The sample size is too small to permit a separate evaluation of the two GVHD regimens utilized in this study. Three patients (UPN 221, 234, and 245) have developed chronic GVHD that is improving with continued therapy with cyclosporine A and prednisone.

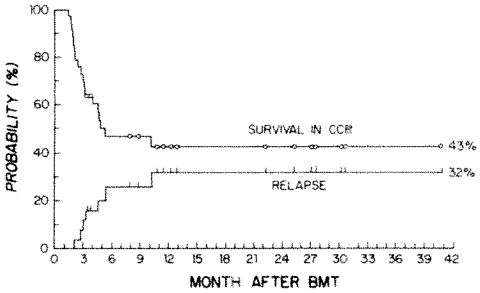

Fig 3. Probability of survival in CCR and probability of relapse after BMT observed in 33 patients with acute leukemia who were treated while not in first remission. Open circles and tick marks denote patients surviving in CCR.

Causes for failure. Fourteen of the 47 patients on study suffered a relapse of their underlying malignancy. Seven patients with acute leukemia relapsed as did seven who had other hematologic malignancies. Five of the relapses occurred in the eight patients who had received single-dose TBI. The earliest relapse was observed 1 month and the latest 10 months after BMT. As illustrated in Fig 3, the actuarial relapse rate for patients with acute leukemia was 32% (2 SEM, 20%). Thirteen patients died of respiratory failure due to interstitial pneumonia that was preceded by an episode of acute GVHD in eight and was associated with a protracted course of sepsis in one patient; the other four patients with interstitial pneumonia did not have acute GVHD. Marrow graft failure after initial engraftment occurred in one patient. The causes of failure are summarized in Table 6.

DISCUSSION

A novel myeloablative/immunosuppressive regimen has been designed and evaluated in a 3-year trial. This investigation has resulted in an excellent survival rate in continued CR of 43% for patients with acute leukemia who were not in first CR when entered onto this study. Our approach differs from previously used preparatory regimens with respect to its chemotherapeutic component in that cyclophosphamide has been replaced by etoposide.

Our pharmacokinetic studies have demonstrated that the drug doses administered correlate well with the attained plasma levels and, more importantly, that etoposide was completely eliminated prior to the time of marrow infusion. It seemed prudent to await total clearance in order to avoid any stem cell injury from residual etoposide.

The toxicity studies carried out in the 47 enrolled patients indicate that high doses of etoposide are well tolerated up to 60 mg/kg body weight. The fact that almost 90% of the

Table 5. Relationship Between Etoposide Dose Levels, Relapse, and Survival

Dose Level (mg/kg)	Relapse (%)	Survival (%)
I. 25-30	1/5 (20)	3/5 (60)
II. 40	4/7 (57)	3/7 (43)
III. 50	0/5 (0)	3/5 (60)
IV. 60	6/23 (26)	11/23 (48)
V. 70	3/7 (43)	0/7 (0)

Table 6. Summary of Disease-Free Survival and Causes for Treatment Failure

	ANLL		ALL		Other Hematologic Malignancies	Total
	IF/RL	CR	IF/RL	CR		
Disease-free survival	5	2	5	3	4	19
Relapse	5	1	0	1	7	14
GVHD & IP	1	1	3	0	3	8
IP	3	0	0	1	0	4
Sepsis & IP	1	0	0	0	0	1
Graft failure	0	1	0	0	0	1
Total patients studied	15	5	8	5	14	47

patients on this study required treatment with systemic broad-spectrum antibiotics is not surprising considering their previous courses of combination chemotherapy and their hematologic status when preparation for BMT was begun.

Based on the toxicities observed, the drug dose of 60 mg/kg was considered the maximum tolerated dose that will also be used in our future studies. In general, regimen-related toxicities have been quite modest; this is best demonstrated by the fact that the earliest death of any patient on this study occurred on day +47 after BMT. The nonleukemic causes for failure were almost exclusively due to expected complications related to allogeneic BMT, ie, interstitial pneumonia in 13 patients, which occurred most frequently after an episode of acute GVHD. The lack of regimen-related long-term toxicities was particularly encouraging.

In combination with an established fractionated TBI method, this regimen has led to a complete response in all patients on this study except for the single case of a child with congenital myelodysplasia characterized by multiple clonal abnormalities (UPN 291). The actuarial relapse rate of 32% can be considered relatively low in comparison with other pretransplant regimens.[1-9] All leukemic recurrences in this study have so far occurred within the first year following BMT, an intriguing observation that warrants longer observations and further studies. Five of the 14 relapses occurred among the eight patients who had received single-dose TBI. These eight patients had been entered into our BMT program on an emergency basis because of their rapidly deteriorating hematologic status. Our limited linear accelerator capacity precluded treatment following the fractionated irradiation regimen. It cannot be decided whether the overall relapse rate would have been lower had those eight patients been treated with the fractionated TBI method.

Our study indicates that the combination of TBI with etoposide is a promising alternative to cyclophosphamide-containing regimens. On the other hand, our investigation leaves a number of problems still unsolved: the two leading causes for failure after any type of preparatory regimen continue to limit posttransplant survival in continued CR, namely, interstitial pneumonia and recurrence of the underlying malignancies. Our study also does not answer the question whether 60 mg etoposide/kg is a more effective dose than, for example, 30 mg/kg. The heterogeneity in hematologic condition and status of the candidates enrolled, the number of patients at the various dose levels, and the length of follow-up does not allow a definite answer to the dose-effect question. However, in view of the toxicity data (Table 4) and the relapse and survival results (Table 5), it seems that 60 mg etoposide/kg is the maximum tolerated dose that is associated with a relatively low relapse rate (26%) and favorable survival (48%). It seems not likely that further dose escalation would result in improved disease-free survival figures.

Finally, it cannot be stated that TBI/etoposide is ultimately better than any of the other previously or currently used preparatory regimens. This issue remains to be dealt with by appropriately designed future prospective randomized studies.

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Erythropoietic Repopulating Ability of Stem Cells From Long-Term Marrow Culture

By David E. Harrison, Charles P. Lerner, and Elaine Spooncer

Hemopoietic precursors are heterogenous with respect to their capacity for self-renewal and long-term repopulating ability. Bone marrow cultures produce a variety of precursors over many weeks, including CFU-S; however, it is important to determine whether these populations retain the functional ability shown by fresh marrow. The most primitive precursor or stem cells have the most long-term repopulating ability. We here describe direct measurements of this ability in cells from marrow cultures by using competitive repopulation assays. Cultured adherent cells repeatedly showed less capacity than fresh marrow cells to repopulate erythropoiesis in irradiated recipients, whereas cultured suspension cells consistently had less capacity than adherent cells. Concentrations of macroscopic CFU-S measured at nine or 12 days were similar in cultured adherent and suspension cells and generally lower than those in fresh marrow. In every experiment, the long-term repopulating ability of the marrow cells used was substantially reduced after transfer into tissue culture. Thus, primitive stem cells may not proliferate in such cultures despite extensive production of CFU-S and more differentiated cell types.

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THE LONG-TERM CULTURE of mouse bone marrow cells is an established method for the prolonged maintenance, proliferation, and differentiation of hemopoietic precursor cells in vitro.[1-6] Analogies with hemopoiesis in vivo include the requirement for the stromal adherent layer that appears to fulfill the role of the hemopoietic environment.[2,3] Furthermore, most cells produced in vitro have been indistinguishable from their in vivo counterparts.[2,4] The hemopoietic precursors produced in vitro that are measured as macroscopic spleen colonies by the CFU-S assay of Till and McCulloch[7] are also identical in many respects to CFU-S from fresh bone marrow.[2] However, the self-renewal capacity of CFU-S from the suspension and, to a lesser extent, of CFU-S from the adherent cells appears to decline greatly during the first 3 weeks in vitro.[6]

Functional heterogeneity of the CFU-S compartment in vivo has previously been well documented by measuring their self-renewal[8,9] or their repopulating ability. Long-term repopulating abilities are low in CFU-S from the peripheral circulation,[10] in previously transplanted marrow cells,[11-13] or following recovery from sublethal irradiation,[13] which shows that the CFU-S assay detects a heterogeneous population of stem cells. Since CFU-S measures are not easy to interpret, we focused on directly testing the primitive stem cell function of long-term repopulation in cultured marrow cells compared with fresh marrow controls.

Repopulating abilities can be measured directly in a sensitive fashion by competitive repopulation.[10-14] Stem cell–depleted recipients were given cells from the various donors to be compared. Each also received a standard dose of competitor stem cells that produced a distinguishable product, in this case hemoglobin.[14] The percentage of donor hemoglobin in the recipient measured the repopulating ability of the donor cells over many months.

MATERIALS AND METHODS

Animal models. C57BL/6J (B6) and $WBB6F_1$ (their F_1 hybrid with WB/ReJ) mice were used. All mice were bred at The Jackson Laboratory, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. These strains possess different alleles at the β chain locus of the hemoglobin molecule and produce hemoglobins that can be separated by standard electrophoretic procedures.[14] Lethally irradiated $WBB6F_1$ recipients were injected with a standard dose of fresh marrow cells (competitor) mixed with the cultured bone marrow cells or fresh marrow controls (donor); the two components of the mixture were of the different genotypes. There is little or no hybrid resistance by $WBB6F_1$ mice against B6 cells.[15]

Long-term marrow cultures. Cultures were established with 6 to 21×10^6 bone marrow cells per flask in Fischer's medium, 20% vol/vol horse serum, and 10^{-6} mol/L hydrocortisone sodium hemisuccinate as described.[1,16] Cultures were maintained at 33°C in 5% CO_2 in air and fed weekly by removing half the growth medium and replacing it with fresh medium.[1] Cells removed in the growth medium were designated cultured suspension cells. Adherent cells from cultures were harvested and pooled by detaching the cells with a rubber policeman.[17]

Marrow and preparation assays. Fresh bone marrow suspensions were prepared by flushing femurs and tibias from donor mice into a buffered balanced salt solution.[11] All cells were filtered through HC3-110 Nytex cloth (mesh opening, 110 μm); then they were counted on a Coulter model ZB1 counter (Coulter Electronics, Hialeah, FL) after the red cells were lysed with Ultralyse (Clay Adams). Recipient mice were irradiated 15 to 18 hours prior to transplantation with a Shepherd Mark I ^{137}Cs irradiator at 220 rad/min. For CFU-S assays, B6 recipients were given 900 R, and $WBB6F_1$ recipients were given 1,000 R. The CFU-S assay was performed according to the method of Till and McCulloch[7]: three to six recipients per group were injected with 10^5 marrow cells in the lateral tail vein and checked after nine or 12 days as noted in the table captions. For competitive repopulation assays, $WBB6F_1$ recipients were given 1,200 R. Three to eight of these mice per group were injected with the following cell numbers, except as noted otherwise in the table caption: 2×10^6 fresh (competitor) B6 marrow cells from a single pool mixed with 1×10^6 from one of the three $WBB6F_1$

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(donor) types—adherent cells or suspension cells from the culture or fresh marrow cells. Donor and competitor genotypes were reversed in one experiment. Seven to 14 weeks later and again 22 to 29 weeks after that, the hemoglobin types were determined in the recipient mice.

RESULTS

Figure 1A shows the numbers of suspension cells and adherent cells in WBB6F$_1$ +/+ bone marrow cultures all made from a single marrow cell pool and tested over a period of 16 weeks. Figure 1B demonstrates that concentrations of CFU-S were equivalent in the suspension cells and adherent layer and that concentrations in fresh bone marrow were consistently higher. The production of these cell types was comparable with other reports.[1-6,17-19] Fig 1C shows data from experiments using the donor adherent and suspension cells from these cultures or fresh WBB6F$_1$ +/+ donor marrow controls; doses of these were individually mixed with a standard dose of fresh B6 marrow competitor cells and injected into irradiated recipients. After 8 to 13 weeks, competitive repopulating abilities were shown by the percentages of donor-type hemoglobin in these recipients. Percentages from cultured cells declined to undetectable levels by 6 weeks of culture, although the adherent cells showed measurable repopulating abilities in subsequent weeks, notably at weeks 8 and 14 when they produced 28% and 32% of the total respectively. Fresh marrow controls consistently repopulated better than the adherent cells and produced 79% and 67% of the total hemoglobin content at weeks 8 and 14.

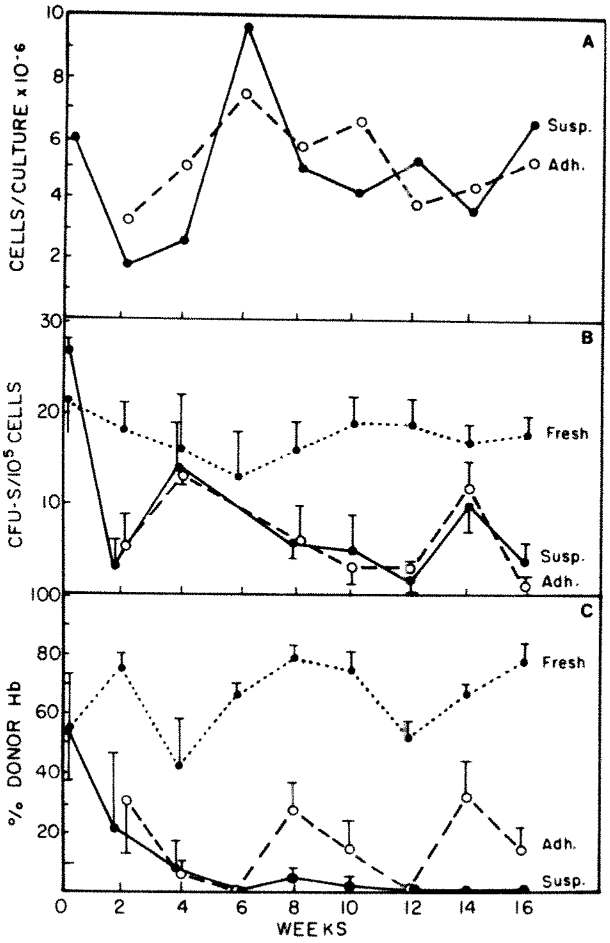

Fig 1. Forty long-term bone marrow cultures were established by using pooled femoral marrow from 3- to 6-month-old WBB6F$_1$ female mice. (A) Cell counts for culture suspension, ●—●; total numbers of cells per adherent layer, O---O, over a 16-week period of culture. (B) CFU-S number/10^5 cells: cultured suspension cells, ●—●; cultured adherent cells, O---O; fresh marrow cells, ●· · · · ·●. Points show mean values, and bars are the standard error for four mice at each point. (C) Percentage of donor-type hemoglobin in recipient mice 8 to 13 weeks after cell mixtures were injected in the competitive repopulation assay. WBB6F$_1$ donor cells were as follows: cultured suspension cells, ●—●; cultured adherent cells, O---O; and fresh marrow cells, ●· · · · ·●. Fresh B6 marrow cells were the competitor. Points show mean values, and bars are the SE for three to four recipients at each point.

Table 1 shows cumulative data over the last 10 weeks for the experiment in Fig 1. Numbers of CFU-S per 10^5 adherent and suspension cells were the same and averaged 31% of fresh marrow values. When recipients were checked 8 to 13 weeks after donor and competitor mixtures were injected, adherent cell–repopulating abilities averaged 20% of those shown by fresh marrow; suspension cells averaged less than 2% of fresh marrow values. When these data were shown in more detail in Fig 1C, adherent cell–repopulating abilities were highly variable. When the same recipients were checked after 22 weeks more (right hand column, Table 1), no donor hemoglobin was detected from cultured adherent cells except those collected at 14 weeks. The average repopulating abilities of adherent cells had dropped to 7% of those shown by fresh marrow, whereas those of suspension cells were undetectable.

Table 2 summarizes data in which genotypes were reversed. The donors were cultured B6 cells or fresh marrow controls that competed with fresh WBB6F$_1$ competitor marrow after 4 and 6 weeks in culture. Once again the adherent and suspension cells from culture had equivalent concentrations of CFU-S, yet the suspension cell–repopulating ability was very low, 4% and 0% of fresh marrow values. Adherent cell–repopulating abilities were 33% and 16% of fresh marrow values.

To determine whether the wide range of repopulating abilities shown by adherent cultured cells resulted from random variations in activities of primitive precursors in the separate culture flasks sampled, four more independent long-term marrow culture experiments were run (Table 3). The initial numbers of marrow cells per flask were about three times higher than those in Fig 1 and Table 1, and the same WBB6F$_1$ +/+ type of marrow donors were used. In assays of long-term repopulating ability by competitive repopulation in experiments 2, 3, and 4 (Table 3), the ratio of donor to competitor cells were 2, 4, and 2.5 times lower respectively for the fresh marrow donor than for the cultured suspension and adherent cells. This compensated for the poorer long-term repopulating abilities of the latter. After 7 to 14 weeks the percentages of donor-type hemoglobin produced by fresh marrow were two to five times higher (after adjusting for donor cell ratios) than percentages produced by cultured adherent cells; the latter competed 1.5 to 2.5 times

Table 1. Erythropoietic Repopulating Ability of Cultured WBB6F$_1$ Marrow

Origin of F$_1$ Donor Marrow	Total Cells/Culture (× 10^{-6})	CFU-S/10^5 Cells	Percentage of Donor-Type Hemoglobin After	
			8-13 wk	30-35 wk
Suspension	5.8 ± 0.9	5.5 ± 1.4 (7)	0.9 ± 0.6 (6)	0 ± 0
Adherent	5.7 ± 0.6	5.0 ± 1.9 (7)	14.3 ± 5.5 (6)	5.5 ± 5.5 (6)
Fresh marrow	—	16.7 ± 0.8 (7)	69.6 ± 4.1 (6)	77.0 ± 4.3 (6)

This table summarizes data shown in Fig 1 that was collected at 2-week intervals during 16 weeks in culture. Cultures were established with 6 × 10^6 pooled WBB6F$_1$ +/+ bone marrow cells per flask, and adherent cells were pooled from three flasks for testing at 2-week intervals. CFU-S data measured at nine days are summarized from 8 to 16 weeks in culture, and cell numbers and the percentage of donor-type hemoglobins are summarized from 6 to 16 weeks in culture. Values for fresh bone marrow are compiled from seven separate experimental time points, each with four recipients for CFU-S, and from six experimental time points for the percentage of donor-type hemoglobin, each with three to four recipients. The right hand columns summarize the percentage of donor-type hemoglobin in the same recipients 8 to 13 and 30 to 35 weeks after donor-competitor mixtures were transplanted. Only the data after 8 to 13 weeks were shown in Fig 1C. The major change after 22 weeks was the absence of hemoglobin produced by adherent cells from 8-, 10-, and 16-week cultures. Those from 14-week cultures continued to produce 33% ± 14% (4) donor hemoglobin compared with 71% ± 7% (3) from the fresh marrow control used at the same time. Data show mean values ± SE (number of experimental time points).

better than cultured suspension cells (Table 3). The advantage of fresh marrow was increased when percentages of donor-type hemoglobin were determined after 24 to 33 weeks in experiments 1 and 2. Numbers of CFU-S were determined at nine days in experiments 1 and 3 and at 12 days in experiments 2 and 4 due to reports that the latter may better represent the primitive stem cell populations.[9] As in previous experiments, CFU-S numbers underestimated the differences in repopulating ability. For example, in experiments 2 and 4, adherent cultured cells had 77% and 97% of the 12-day CFU-S as fresh marrow but 58% and 49% of the donor hemoglobin type at 7 to 14 weeks (Table 3).

DISCUSSION

The primitive stem cell function of competitive repopulation against fresh marrow was substantially reduced in adherent cells from long-term marrow cultures and further reduced in suspension cells. These findings are consistent with the results of Mauch and colleagues[6] who reported that CFU-S self-renewal capacities of suspension cells were extremely low and those of adherent cells were about threefold lower than those of fresh marrow from weeks 3 to 8 of culture. Schofield and Dexter[18] also found very poor self-renewal in CFU-S from the suspension. However, they found normal self-renewal in CFU-S from adherent cells, a conflicting result perhaps due to their use of spleen colonies in studying self-renewal. The most primitive stem cells may repopulate the marrow, not the spleen, of an irradiated recipient. Marrow precursor cells are studied by the long-term repopulation experiments in this report, and marrow was the source of cells whose self-renewal was studied by Mauch and colleagues.[6] The conclusion that stem cells in the adherent layer are less primitive than those in fresh marrow is supported by the loss of adherent cell–repopulating abilities between 7 and 14 weeks and 24 and 35 weeks in the same recipients (Tables 1 and 3). This also illustrates the value of measuring competitive repopulation over the long term so that measures are likely to represent truly primitive stem cells, although only erythrocyte function is assayed, and the possibility that erythroid rather than multipotential stem cells are measured is not formally eliminated.

The apparent reemergence of adherent stem cells with considerable long-term repopulating abilities after 14 weeks of culture in our studies (Fig 1C, Table 1) probably represents variability in individual cultures since cells were pooled from three separate (randomly selected) cultures for each time point. When more experiments were done and the cultures were started with about three times higher numbers of marrow cells, the relative repopulating abilities of cultured adherent compared with fresh marrow cells varied from about 20% to 50% (Table 3). This variability probably reflects the rarity of the true primitive stem cell and the random nature of its preservation among the bone marrow cells used to establish a culture.

Table 2. Erythropoietic Repopulating Ability of Cultured B6 Marrow

Origin of B6 Donor Marrow	Culture Duration (wk)	Total Cells/Culture (× 10^{-6})	CFU-S/10^5 Cells	Percentage of Donor-Type Hb*
Suspension	4	2.1	17.7 ± 0.3 (4)	0
Adherent	4	1.9	13.5 ± 0.5 (4)	11.5 ± 4.5 (2)
Suspension	6	5.9	3.4 ± 1.0 (3)	1.3 ± 1.3 (3)
Adherent	6	6.1	3.5 ± 0.3 (3)	5.3 ± 3.9 (4)
Fresh marrow	—	—	16.8 ± 2.3 (8)	33.0 ± 3.0†

Long-term bone marrow cultures were established from 10 to 15 × 10^6 pooled B6 femoral marrow cells per flask. After 4 and 6 weeks, cultured suspension and adherent cells were harvested. Their nine-day CFU-S were measured, and for the competitive repopulation assay, recipient mice were injected with 1 × 10^6 fresh WBB6F$_1$ competitor marrow cells from a single pool mixed from 2 × 10^6 suspension, adherent, or fresh B6 (donor) marrow cells. Data show mean values ± SE (number of recipients).

*Percentage of donor type hemoglobin in recipient mice 9 to 11 weeks after mixtures injected for the competitive repopulation assay.

†This value was determined from three separate experiments with four recipients each.

Table 3. Erythropoietic Repopulating Ability of Cultured WBB6F$_1$ Marrow Prepared in Four Separate Experiments

Origin of Donor Marrow	Culture Duration (wk)	Total Cells/Culture ($\times 10^{-6}$)	CFU-S/10^5 Cells	Percentage of Donor-Type Hemoglobin After	
				7-14 wk	24-33 wk
Experiment 1					
Suspension	8	11.1	2.9 ± 0.5	8 ± 8 (3)	0 ± 0 (3)
Adherent	8	9.0	4.4 ± 0.7	19 ± 5 (7)	15 ± 7 (7)
Fresh marrow	—	—	15.0 ± 1.2	89 ± 1 (4)	87 ± 5 (4)
Experiment 2					
Suspension	8	9.1	7.7 ± 0.4*	37 ± 12 (7)	49 ± 16 (4)
Adherent	8	4.3	8.7 ± 0.4*	72 ± 6 (9)	78 ± 6 (8)
Fresh marrow (×2)†	—	—	11.3 ± 0.8*	62 ± 4 (3)†	89 ± 4 (3)†
Experiment 3					
Suspension	8	2.3	7.8 ± 0.8	48 ± 9 (4)	—
Adherent	8	1.5	10.9 ± 1.0	68 ± 3 (4)	—
Fresh marrow (×4)†	—	—	17.8 ± 1.3	67 ± 6 (5)†	—
Experiment 4					
Suspension	4	7.3	12.5 ± 1.6*	29 ± 12 (4)	—
Adherent	4	4.6	20.3 ± 1.2*	82 ± 1 (3)	—
Fresh marrow (×2.5)†	—	—	21.8 ± 1.6*	67 ± 3 (5)†	—

Long-term bone marrow cultures were established from pooled WBB6F$_1$ +/+ femoral marrow cells using 21 × 10^6, 15 × 10^6, 16 × 10^6, and 19 × 10^6 cells per flask in experiments 1, 2, 3, and 4, respectively. After 4 to 8 weeks, cultured suspension and adherent cells were harvested. Their CFU-S were measured after nine or 12 days along with those of a fresh marrow control F$_1$ +/+ donor in three to six recipients each. For the competitive repopulation assay in experiment 1, recipient mice were injected with 2.0 × 10^6 fresh B6 competitor cells from a single pool mixed with 1 × 10^6 suspension, adherent, or fresh F$_1$ donor cells.

*Twelve-day CFU-S were measured in experiments 2 and 4, whereas nine-day CFU-S were measured in experiments 1 and 3.

†In experiments 2, 3, and 4, the ratios of the donor to competitor cells were, respectively, 2, 4, and 2.5 times lower for the fresh marrow control than for the cultured cells. Thus in experiment 2, 1.0 × 10^6 cultured donor cells were mixed with 1.0 × 10^6 competitor cells, and 1.0 × 10^6 fresh donor cells were mixed with 2.0 × 10^6 competitor cells. In experiment 3, 2.0 × 10^6 cultured donor cells were mixed with 1.0 × 10^6 competitor cells, and 1.0 × 10^6 fresh donor cells were mixed with 2.0 × 10^6 competitor cells. Experiment 4 was like experiment 2 except that 1.0 × 10^6 fresh donor cells were mixed with 2.5 × 10^6 competitor cells.

The consistent reduction in repopulating capacity of adherent cells may also be due to the mechanical disaggregation of hemopoietic tissue that occurs during culture establishment. Indeed, cells from the adherent layers of our cultures repopulated at least as well as marrow cells that had undergone one in vivo transplantation[11-13] in which analogous disruption of the tissue occurs, although it should be noted that at least 100 times more proliferation occurs in vivo. Both in vivo and in vitro, the stress of proliferation and differentiation may push the cells of highest repopulating ability to form cells with lower levels of repopulating ability. It is unlikely that the defects in cultured cells result from the absence of essential accessory cells or the presence of suppressing cells because both donor and competitor cells are grown in the same recipient in the competitive repopulation assay.

The superior repopulating ability and self-renewal of adherent cultured cells compared with cells in the suspension demonstrate why the stroma is essential to the prolonged duration of hemopoiesis in vitro[1-3,19] and confirm findings that the bulk of cells in the suspension are produced in and released from the adherent layer.[20] Although human bone marrow cells do not grow for prolonged periods in vitro, they produce cultures with adherent layers within which the more primitive multipotential cells are preferentially located.[21] Even cultured marrow suspension cells have been shown to save the lives of lethally irradiated mice.[22] However, any future use of human cultured cells for transplantation would best be performed by using cells of the adherent layer to enhance chances of long-term recovery.

Measurement of the number of CFU-S in cultured cells was not proportional to their ability to repopulate hemopoiesis. This was true in those measured at both nine and 12 days (Tables 1 to 3), although the latter are reported to show more primitive stem cell functions. Thus, quantitation by the CFU-S assay of stem cells grown in vitro does not necessarily reflect the maintenance of primitive stem cells. In order to grow the most primitive stem cells in vitro, procedures should be developed that increase the long-term repopulating abilities of the cultured cells. In the six separate experiments reported here, the repopulating abilities of cultured cells were always less than those of fresh marrow, and the total numbers of cells were much lower than the numbers of marrow cells used to initiate the cultures (Tables 1 to 3). Thus the cells responsible for repopulating ability do not appear to increase in long-term marrow cultures. Nevertheless, the cells of the adherent layer evidently provide a useful model in vitro for studying hemopoiesis in the bone marrow, especially that occurring after a single transplantation. In contrast, the CFU-S released into the suspension phase may be more functionally comparable with those in peripheral blood than in marrow.

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The Hematologic Characteristics of Sickle Cell Anemia Bearing the Bantu Haplotype: The Relationship Between $^{G}\gamma$ and HbF Level

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Previous work has demonstrated that the HbS gene has appeared and expanded three times in Africa in three separate geographic locations and that these three distinct mutational events can be identified by linked DNA polymorphic sites (haplotypes) surrounding the abnormal gene. We have reported that the Senegalese and Beninian haplotypes differ in $^{G}\gamma$ expression, mean percentage of HbF, and percentage of dense cells. We now report on the third haplotype, the Bantu, and find that it has intermediate features, namely, the high mean percentage of HbF and low percentage of dense cells associated with the Senegalese haplotype, but with a low percentage of $^{G}\gamma$ expression similar to the Beninian haplotype. The distribution of percent HbF is quite different from Senegal haplotype-bearing sickle cell anemia patients since it covers a much wider range. The low $^{G}\gamma$ expression is also different from the Beninians since it contains a significant and unique cluster of individuals with lower than 38% $^{G}\gamma$. Interestingly, among the Bantu there is a strong correlation between HbF levels and $^{G}\gamma$ expression, which is not seen with the other haplotypes. These findings open the possibility that among the Bantu haplotype-bearing individuals two chromosomal types exist that define different levels of $^{G}\gamma$ and HbF expression. Further structural exploration of these two potential subhaplotypes is needed.

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PRESENT EVIDENCE suggests that the HbS gene appeared in Africa at least three times and presumably expanded in frequency through the selective pressure of *Plasmodium falciparum* malaria. The best evidence for this hypothesis comes from the geographically segregated linkage disequilibrium of the HbS gene with three different β gene cluster haplotypes (the Senegal, the Benin, and the Bantu haplotypes).[1] That these are indeed the major haplotypes associated with the HbS gene is confirmed by the study of Jamaican and American sickle cell anemia (SS) patients.[2,3] This finding suggested the possibility that the HbS gene could be accompanied by a different set of linked epistatic genes in each of these three haplotypes. Indeed, we have reported previously that SS patients bearing the Senegalese haplotype have more HbF, less dense cells, and higher $^{G}\gamma$ expression than patients in which the HbS gene is linked to the Benin haplotype.[4] We report here on the hematologic and genetic characteristics of the third haplotype, the Bantu, and a comparison with the other two reveals that it is associated with yet another unique set of features.

METHODS

Samples were obtained, after informed consent, from all the unrelated attendants of the outpatient clinic who came without regard to present symptomatology after a public announcement. Patients with a history of painful crisis less than a week old were eliminated from the sample. The hematologic parameters were determined as previously described.[4] β Gene cluster and α gene haplotypes were determined by Southern blotting in the manner reported elsewhere[1] by using the *Hind*III detectable polymorphic site in the IVSII region of the γ genes, the *Hinc*II sites in the $\psi\beta$ gene region, and *Hpa*I site 3' to the β gene. In a subset of samples in addition, the *Hinc*II site 5' to ϵ, *Xmn*I 5' to γ, the *Pvu*II site 5' to $\psi\beta$, the *Ava*II in IVSII of the β chain and the *Bam*HI 3' to the *Hp*I site were determined. The α haplotype was determined by *Bam*HI digestion. Percoll-Stractan isopycnic gradients were made as described previously,[5] and the four classes of cells were defined as fraction 1 (Fl, mean corpuscular hemoglobin concentration [MCHC] less than 33 g/dL, densities less than 1.083 g/mL), fraction 2 (F2, MCHC between 33 and 37 g/dL, densities between 1.083 and 1.093 g/mL), fraction 3 (F3, MCHC between 37 and 42 g/dL, densities between 1.093 and 1.105 g/mL), and fraction 4 (F4, MCHC greater than 42 g/dL, densities greater than 1.105 g/mL). Fl contains reticulocytes and young cells, F2 reversible discocytes, F3 the unsicklable SS discocytes, and F4 the densest irreversible discocytes and the irreversibly sickled cells (ISC).

Percent HbF present as $^{G}\gamma$ was determined by a high-performance liquid chromatography method using a Vydac large-pore (300 Å) C_4 column, 4.6 × 250 mm (Separations Group, Hesperia, CA) and an acetonitrile/trifluoroacetic gradient. The HbF was prepurified by BioRex-70 (Bio-Rad, Richmond, CA) column chromatography.

Statistics were done with the help of an IBM-XT computer and the Statgraphics program (STSC-Plus-Ware, Rockville, MD) for plotting, frequency histogram, regression analysis, the two-sample *t* test, and cluster analysis.

RESULTS

We have studied 64 unrelated SS patients from the Central African Republic. Of these 128 chromosomes, 116 β genes were linked to the Bantu haplotype (90.6%), whereas 12 chromosomes were atypical and their characteristics will be reported elsewhere.

Hematologic and genetic characteristics of SS-bearing Bantu haplotype. We report here on the hematologic and genetic characteristics of 54 SS patients (mean age, 14 ± 6.3 SD) in whom the Bantu β gene cluster haplotype[1] has been demonstrated by defining five polymorphic sites. Of these 54 patients, 36 were females and 18 males, 26 were $\alpha\alpha/\alpha\alpha$

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(48.4%), 22 were $-\alpha/\alpha\alpha$ (40.6%), and 6 were $-\alpha/-\alpha$ (11.1%). The gene frequency of the $-\alpha$ haplotype is then 0.315 among these patients, higher than that found among AA individuals in the Central African Republic,[6] which predicts that about 62% of patients with SS have concomitant α-thalassemia. This result is in agreement with earlier data that found the frequency of $-\alpha$ increased among sickle cell anemia patients as compared with the normal population,[7,8] which suggests a protective effect of α-thalassemia in terms of survival. This suggestion has been confirmed in several populations by the age dependence of the $-\alpha$ gene frequency,[6-8] although the data of others[9] have only partially confirmed this finding by observing four homozygotes among the patients over 50 years old.[10]

In Table 1 the hematologic and genetic characteristics of the total sample and of the sample separated according to α gene status are depicted. Prominent among the findings are the high percentage of HbF (10.4%) and the corresponding low percentage of F4 (8.9%) and ISC (8.7%). The HbF levels are significantly correlated with the level of F4 ($r = .320$, $P < .018$, n = 54) as previously demonstrated for SS patients with the Senegalese haplotype.[4] HbF also correlated with ISC ($r = .299$, $P < .027$), not surprisingly since the percentage of ISC in the total population correlates with the percentage of F4 very strongly ($r = .863$, $P < 4.4 \times 10^{-17}$). It is interesting to note that the correlation of the percentage of ISC *v* F4 had an r^2 of .86 for $\alpha\alpha/\alpha\alpha$ individuals but that the r^2 for $-\alpha/\alpha\alpha$ and $-\alpha/-\alpha$ patients was much lower (.32), which suggests that in concomitantly α-thalassemic individuals the percentage of ISC is a less accurate reflection of the percentage of dense cells.

The reticulocyte count was correlated with F4 in the total sample ($r = .304$, $P < .025$). The hematocrit (Hct) value was marginally correlated with the percentage of HbF ($r = .26$, $P < .05$) and the reticulocyte count ($r = .26$, $P < .05$).

The $^G\gamma$ ratio was 40.4% (±6.2 SD) in 54 patients studied, and no effect of α-thalassemia on this parameter is observed.

Table 2. Comparison of the Hematologic Characteristics of the Three Major Haplotypes Linked to HbS

Characteristics	Senegal*	Benin*	Bantu
n	28	41	53
Hct (%)	24.6 ± 3.1	24.2 ± 4.9	23.9 ± 3.7
HbF (%)	12.3 ± 5.3	8.0 ± 5.2	10.4 ± 4.9
F4 (%)	12.2 ± 8.8	17.0 ± 8.9	8.9 ± 8.1
ISC (%)	9.3 ± 5.3	14.3 ± 10.3	8.7 ± 9.4
$^G\gamma$ (%)	65.8 ± 8.9	44.8 ± 4.6	40.4 ± 6.2

Data are means ± SD. See the text for significance.

*Data from reference 4.

Table 2 compares the hematologic properties of the Bantu haplotype SS with the Senegalese and Beninians. The Bantu and the Senegalese share a high percentage of HbF and a low percentage of F4, even when the elevated frequency of α-thalassemia in the Bantu population is taken into account by comparing the non-α-thalassemic Bantu to the Senegalese in whom α-thalassemia is less prevalent. Nevertheless, they are different in that the Senegalese haplotype is linked to high $^G\gamma$ expression and the Bantu is linked to low $^G\gamma$ expression in the adult. Both haplotypes are in turn very different from the Beninian haplotype, which is characterized by low HbF and high F4 percentages.

The percentage of HbF among the Bantu is significantly different from the Beninian ($P < .009$), as are the percentage of F4 ($P < .00002$) and the percentage of ISCs ($P < .005$). The Bantu percentage of F4 was also significantly different from the Senegalese percentage of F4 ($P < .05$).

The distribution of the percent HbF among patients with sickle cell anemia is also different for the three haplotypes (Fig 1). The mode for the percentage of HbF among patients with the Benin SS haplotype is in the class between 2.6% and 5%, with a nongaussian distribution trailing off to higher percentages. In contrast, the mode for Senegal and Bantu haplotypes is 10.1% to 12.5%, although a higher proportion (33% *v* 25%) of Senegalese are found with a percentage of HbF greater than 10. The difference between these two groups is the lack of Senegalese SS with a HbF below 5%, a class that is populated by over 11% of the Bantu SS patients.

The relationship between percent HbF and percent $^G\gamma$. Figure 2 illustrates the relation between the percentage of $^G\gamma$ and the percentage of HbF for the patients bearing the Bantu haplotype and a comparison with the Senegalese and Beninian sickle cell anemia patients. Not all Senegalese SS are known to carry the Senegalese haplotype. For the Bantu SS restricted to individuals 5 years of age or older (n = 52), the

Table 1. Hematologic and Genetic Characteristics of Sickle Cell Anemia Bearing the Bantu Haplotype

Characteristic	All SS	SS + $\alpha\alpha/\alpha\alpha$	SS + $-\alpha/\alpha\alpha$	SS + $-\alpha/-\alpha$
n	54	26	28	6
Sex, percent female	66.6	57.61	75.0	66.6
Age	14.0 ± 6.3	12.6 ± 6.8	15.4 ± 5.7	14.7 ± 8.4
Hct	23.8 ± 3.7	23.1 ± 3.3	24.5 ± 3.9	24.8 ± 4.0
Reticulocytes (%)	25.0 ± 13.1	25.5 ± 13.7	24.8 ± 12.8	19.6 ± 9.9
HbF (%)	10.4 ± 4.8	10.35 ± 5.1	10.4 ± 4.7	10.8 ± 0.7
$^G\gamma$	40.4 ± 6.2	42.6 ± 5.0	39.2 ± 7.4	37.5 ± 1.4
ISC	8.7 ± 9.4	8.8 ± 12.0	8.5 ± 6.1	7.5 ± 4.1
F1*	46.9 ± 22.5	50.5 ± 26.3	43.5 ± 18.2	43.7 ± 24.9
F2	39.7 ± 19.2	34.9 ± 21.2	44.1 ± 16.2	45.8 ± 22.3
F3	4.5 ± 3.3	4.7 ± 3.7	4.4 ± 2.9	4.4 ± 3.1
F4	8.9 ± 8.0	9.9 ± 10.5	7.9 ± 4.7	6.0 ± 3.0

Numbers (except for n = number of patients and sex) are mean ± SD.

*F1, F2, F3 and F4 are density-defined red cell SS fractions obtained from Percoll-Stractan isopycnic gradients.

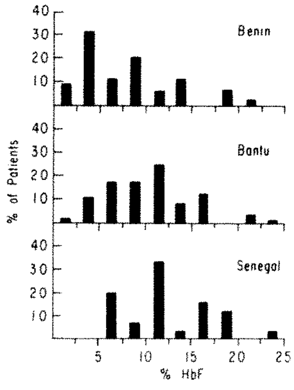

Fig 1. Frequency histogram of the percentage of HbF among SS patients bearing the Benin, Bantu, and Senegal haplotypes.

regression coefficient of percent HbF *v* percent $^{G}\gamma$ was 0.71 with a slope of 0.53 and a $t = 7.066$, $DF = 49$, and $P < .0001$. The presence or absence of α-thalassemia does not affect the results. Statistical cluster analysis of the data in Fig 2A reveals that there is a cluster of patients with less than 38% $^{G}\gamma$, scarcely represented among Beninian patients (2B). When the percent HbF in the group of Bantu SS patients with less than 38% $^{G}\gamma$ is compared with the percent HbF in those with more than 38.1%, the two-sample *t* test is highly significant, with a mean of 6.34 ± 2.46, and 12.46 ± 4.0, respectively, ($t = 5.909$, $DF = 50$, $P < 3.0 \times 10^{-7}$ (Table 3). In addition, the variance in the percent HbF among those above 38.1% $^{G}\gamma$ is much higher than that in those below 38% $^{G}\gamma$. The presence or absence of α-thalassemia, again, does not affect these findings. No differences in the mean of the Hct, reticulocyte count, F4, or ISC count was observed between these two subsets. The low percentage of F4 and ISC (despite the lower percentage of HbF) in the $<38\%$ $^{G}\gamma$ is due to a high proportion of α-thalassemics among these 18 individuals. In addition, full haplotypes (ten polymorphic sites) were determined in three subjects with less than 38% $^{G}\gamma$ and three with greater than 38% $^{G}\gamma$; no differences were observed, and all subjects were *Xmn* I-negative.

Most interesting are the calculations of regression curves below and above 38% $^{G}\gamma$. The *r* value for a correlation between HbF *v* ISC or F4 is (not significant) in the subset equal or less than 38% $^{G}\gamma$, whereas it is highly significant (for ISC = $r = -.53$, $DF = 29$, $t = -3.32$, $P < .005$; and for F4 = $r = -.45$, $DF = 29$, $t = -2.79$, $P < .01$) among those equal or higher than 38.1% $^{G}\gamma$.

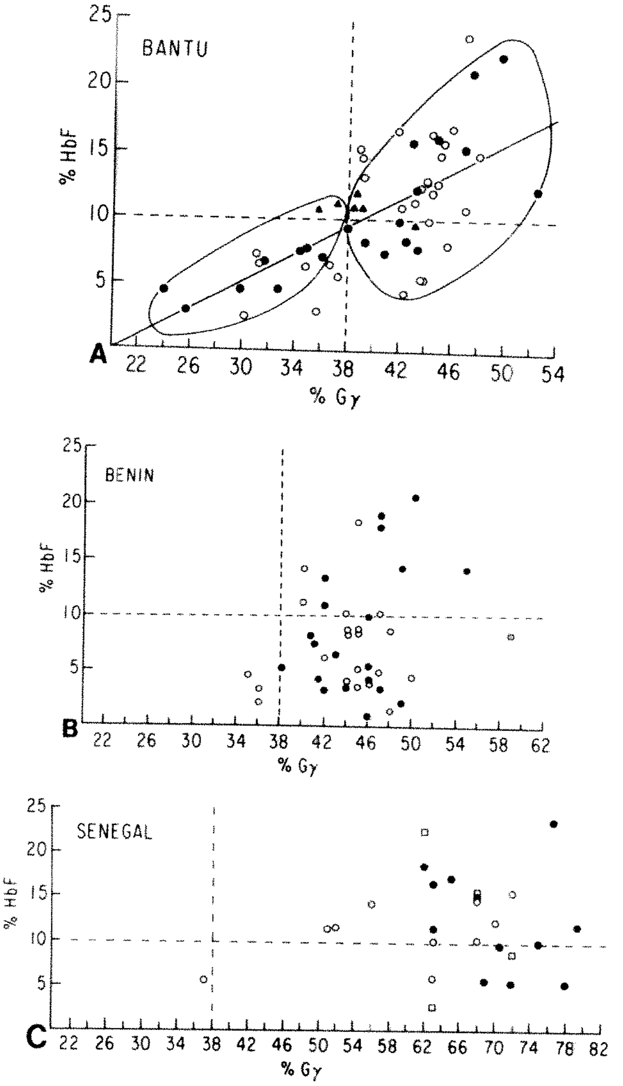

Fig 2. (A) Scatter plot of percent HbF *v* percent $^{G}\gamma$ for Bantu haplotype SS patients. ●, $\alpha\alpha/\alpha\alpha$; ○, $-\alpha/\alpha\alpha$; ▲, $-\alpha/-\alpha$. The straight line represents regression of the percentage of HbF on $^{G}\gamma$, which has an $r = .71$, slope $= 0.53$, $t = 7.066$, $DF = 49$, and $P < .0001$. Curved lines are clusters derived from a matrix that contains percent $^{G}\gamma$, percent HbF, and percent F4 using a Statgraphics program with the restriction that two clusters be formed. (B) Scatter plot of percent HbF *v* percent $^{G}\gamma$ for Beninian haplotype SS patients. ●, $\alpha\alpha/\alpha\alpha$; ○ $-\alpha/\alpha\alpha$. No significant correlation was found. (C) Scatter plot of percent HbF *v* percent $^{G}\gamma$ in SS patients originating in Senegal. ●, Senegal haplotype; □, atypical haplotype; ○, unknown haplotype. No significant correlation was found.

DISCUSSION

One of the most perplexing features of sickle cell anemia is the extreme variability of the hematologic characteristics and severity of its symptomatological components when a large number of patients are analyzed. It has recently become clear that one of the sources of this heterogeneity is the presence of the HbS gene on chromosomes bearing at least three different arrays of linked genes and sequences, which arose and expanded in geographically segregated regions of Africa and generated three β gene cluster haplotypes linked to these HbS genes.[1] The Senegalese haplotype is found in Senegal and surrounding areas of Atlantic West Africa; the Benin haplotype is found in Benin and surrounding areas of Central West Africa and in North Africa and the Mediterranean where its presence has been extended through gene flow; and finally, the Bantu haplotype is found in the Central African Republic and presumably (on the basis of *Hpa*I polymorphism data) in the whole of the Bantu-speaking equatorial and Southern Africa.

To what extent do these different haplotypes linked to HbS generate different hematologic, genetic, and clinical features? Are there three types of sickle cell anemia?

Table 3. Hematologic Parameters in SS Patients with Bantu Haplotype (≥5 Years Old) Divided into Two Subsets by $^G\gamma$ Level

	<38% $^G\gamma$*		>38.1% $^G\gamma$†	
	Mean ± SD	Variance	Mean ± SD	Variance
HbF (%)	6.3 ± 2.5	6.1	12.5 ± 4.0	16.0‡
Hct (%)	25.2 ± 3.6	13.2	23.0 ± 3.7	13.4
Reticulocytes (%)	26.1 ± 13.9	192.7	25.3 ± 13.3	177.6
F4 (%)	8.1 ± 5.5	30.6	9.6 ± 9.3	86.6
ISC (%)	7.8 ± 6.0	36.1	9.7 ± 11.2	125.9

*7($\alpha/\alpha\alpha$), 9($-\alpha/\alpha\alpha$), 2($-\alpha/-\alpha$); n = 18.

†20($\alpha\alpha/\alpha\alpha$), 14($-\alpha/\alpha\alpha$); n = 34.

‡$P < .0000003$.

We have partially answered this question, previously, by studying[4] haplotypically homozygous SS patients bearing the Senegalese and the Beninian haplotypes. That study demonstrated that, indeed, the Senegalese haplotype linked to the HbS was characterized by higher HbF levels, a lower percentage of dense cells, and a high $^G\gamma$ expression in the adult. That their features are genetic in origin is given considerable support by the finding that the same haplotype induces a similar HbF response in thalassemia even though this disease is under different pathophysiological control.[11] In contrast, the Beninian haplotype was characterized[4] by SS patients exhibiting lesser amounts of HbF, a larger proportion of dense cells,[12] and low $^G\gamma$ gene expression. A model that describes the combined effect of α-thalassemia and percent HbF on percent F4 (dense cells) was developed for our New York black population[8] and for the Senegalese and Beninian populations.[4] The relative contribution of α-thalassemia and percent HbF was different for each population as would be anticipated if there were a third factor (haplotype) that also affects dense cell generation.

The characteristics of SS patients bearing the Bantu haplotype have features that distinguish them from the other two haplotypes. First, the mean and mode (Table 2 and Fig 1) of the percentage of HbF is high and similar to the SS patients with Senegalese haplotypes. Nevertheless, the distribution of the values is different: no SS patient with Senegalese haplotype had an HbF level less than 5%, whereas 11% of Bantus had an HbF level between 0% and 5%, although mostly between 2.6% and 5% (Fig 1). The situation is considerably different among the patients bearing the Benin haplotype who exhibit a HbF distribution with a mode between 2.6% and 5% and a very nongaussian distribution with a long tail toward the higher values. Another important feature of the Bantu sickle cell anemia patient is their low percentage of dense cells, again similar to the Senegalese and not exclusively due to higher frequency of α-thalassemia among the Bantu. Although there is a significant correlation between percent F4 or percent ISC and percent HbF in both populations, a lesser proportion of the variability can be explained by the level in the percentage of HbF among the Bantu than in the Senegalese ($r^2 = .53$ v .10). However, if only the non-α-thalassemic Bantu patients are considered, r^2 rises from .10 to .22 with a $P > .02$.

A further characteristic that differentiates the Senegalese from the Bantu is the $^G\gamma$ expression. The Senegalese exhibit a high $^G\gamma$ expression among adults, an infrequent finding among normal individuals who switch from the newborn 70% $^G\gamma$/30% $^A\gamma$ ratio to the adult 40% $^G\gamma$/60% $^A\gamma$ ratio during the first 4 months of life. The Bantu SS have the low $^G\gamma$ expression (Table 1) characteristic of adults.

Although the genetic linkage between HbS and haplotypes and between haplotypes and $^G\gamma$ expression is well established,[1,4,11] no formal linkage analysis of the percentage of HbF and haplotype has yet been possible. Although the statistically significant association between the mean and frequency distribution of the percentage of HbF and haplotype suggests genetic linkage, it is nevertheless already clear that modulating epistatic effects from other genes or factors will result in a not very tight linkage. Family studies, hampered by the absence of the anemia-induced HbF response in parents, will be needed to quantify the linkage.

The next question is whether there is a correlation between percent $^G\gamma$ and percent HbF. Previous studies of American SS patients over the age of 5 have found no significant correlation between these two parameters[13] (although a weak correlation was found in SS patients below this age), and the same is true of the previously studied Beninian SS patients[4] (Fig 2). On the other hand, among SS patients bearing the Bantu haplotype there is a significant correlation ($P <$ .0001) between $^G\gamma$ and HbF that, on further analysis, seems to arise from a cluster of individuals with a low expression of $^G\gamma$ (<38%). This group is little represented among the Senegalese, the Beninians, and the patients analyzed by Huisman et al.[13] The low $^G\gamma$ subset is associated with a low percentage of HbF, whereas the subset with higher $^G\gamma$ expression has a significantly higher percentage of HbF (Table 3). Interestingly, the variance is low in the former and large in the latter. These findings explain why no significant correlation has been found in populations of patients in which the group with low $^G\gamma$ expression is not well represented. In addition, if the data of the Huisman group[13] is divided into less than and more than 38% $^G\gamma$, a significantly lower percentage of HbF is observed for the <38% $^G\gamma$ subset: for patients who are 5 years old or older, the mean is 3.87 ± 3.3 compared with 12.2 ± 5.5 for the >38.1% $^G\gamma$ ($P < .01$).

The low $^G\gamma$ subset exhibits poor correlation between HbF and F4 or ISC, whereas the higher than 38.1% subset exhibits a strong negative correlation ($r = .53$, $P < .005$). This dimorphic population explains the lower correlation of HbF and dense cells in the overall sample.

The heterogeneity in $^G\gamma$ expression among the Bantu and its relation to HbF expression is a significant finding that opens new avenues of investigation. Our working hypothesis is that the subset with lower than 38% $^G\gamma$ corresponds to a distinct chromosome bearing different HbF determinants than the rest of Bantu haplotypes (although not distinguishable by the ten endonuclease-definable polymorphic sites including the *Xmn*I site). Efforts to define these determinants by sequencing are underway in our laboratory.

A complementary explanation is that the enrichment factor (the ratio between F cells and F reticulocytes[14]) might modulate the HbF level, particularly among individuals with a percentage of $^G\gamma$ between 38 and 50. This last effect might be the consequence of the dependence of the enrichment factor on the constitutive HbF expression (percentage of HbF reticulocytes), a possibility supported by the data of Dover et al.[14] These variations also might affect the resulting percentage of HbF more significantly in higher HbF-producing individuals. In addition, the variability of dense cells with respect to HbF level might depend on the distribution function of HbF among HbS-containing cells, a factor that may be genetically determined.

We conclude that the Bantu haplotype results in yet another set of hematologic and genetic characteristics that exhibit a high mean HbF percentage (although with a different distribution than the Senegalese), a low percentage of dense cells (although less determined by the HbF level than among Senegalese), and a low $^G\gamma$ expression (unlike the high $^G\gamma$ expression characteristic of the Senegalese) but with a wide range of the $^G\gamma$ percentage (unlike the distribution among Beninians).

Because patients in the Americas originated from the three geographically segregated but haplotypically homogenous areas of Africa,[15] they can be expected to belong to the three types of sickle cells anemia, but it is further complicated by both non-African admixture and by the presence of a high number of haplotypically heterozygous individuals whose hematologic characteristics have not been defined.

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We are indebted to the staff of the University Hospital at Bangui, the dean and staff of the Medical School at Bangui, the Sickle Cell Clinic founded by the late Dr H. Baya, and the members of the Institute Pasteur for considerable help and assistance in the completion of our project.

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The In Vitro Synthesis of Polypeptides for the Platelet Membrane Glycoproteins IIb and IIIa

By Samuel M. Silver, Margaret M. McDonough, Gaston Vilaire, and Joel S. Bennett

The platelet membrane glycoproteins IIb (GpIIb) and GpIIIa form calcium-dependent heterodimers containing binding sites for fibrinogen, von Willebrand factor, and fibronectin. Although GpIIb and GpIIIa are distinct proteins, both GpIIb and GpIIIa are deficient in platelets from individuals with the recessive disorder Glanzmann's thrombasthenia. To gain a better understanding of the genetic basis for GpIIb and GpIIIa synthesis, we studied their synthesis by two human leukemia cell lines, HEL and K562. HEL cells contained complexes of GpIIb and GpIIIa, and K562 cells expressed GpIIIa, but not GpIIb, when stimulated with phorbol-12-myristate-13-acetate (PMA). RNA from HEL cells directed the in vitro synthesis of a 110,000-M_r precursor for GpIIb and a 92,000-M_r precursor for GpIIIa, which indicates that the synthesis of GpIIb and GpIIIa by HEL cells is directed by separate mRNAs. In contrast, RNA from PMA-stimulated K562 cells only directed the synthesis of a 92,000-M_r precursor for GpIIIa. The dissociation of GpIIb and GpIIIa synthesis in K562 cells suggests that GpIIb and GpIIIa may be the products of separate genes.
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THE GLYCOPROTEINS IIb and IIIa (GpIIb and GpIIIa)[1] exist as calcium-dependent heterodimer complexes on the surface of unactivated platelets. Following platelet activation, the complexes express binding sites for fibrinogen,[1,2] von Willebrand factor,[3] and fibronectin.[4] GpIIb and GpIIIa are distinct proteins. They differ in subunit structure, have dissimilar tryptic peptide maps, and lack antigenic cross-reactivity.[5-7] Thus, it is not obvious why both glycoproteins are deficient to a similar extent in platelets from individuals with the autosomal recessive disorder Glanzmann's thrombasthenia.[8] To gain a better understanding of the genetics of GpIIb and GpIIIa, in vitro studies of their synthesis will be required. Unfortunately, platelets are not suitable cells for these studies because they retain little protein synthetic capacity. Furthermore, although megakaryocytes synthesize GpIIb and GpIIIa, the number of megakaryocytes that can be isolated is limited by their paucity in bone marrow specimens.[9,10] To overcome these problems we have studied GpIIb and GpIIIa synthesis using cells from two human leukemia cell lines, HEL and K562.[11,12] In this report we have characterized the GpIIb and GpIIIa synthesized by HEL and K562 cells. Furthermore, using RNA isolated from each cell line, we have demonstrated that the synthesis of these proteins is directed by separate species of mRNA, which suggests that GpIIb and GpIIIa are the products of separate genes.

MATERIALS AND METHODS

Preparation of polyclonal antisera against platelet GpIIb and GpIIIa. Polyclonal anti-GpIIb and anti-GpIIIa antisera were prepared by immunizing rabbits with purified human platelet GpIIb and GpIIIa. To purify platelet GpIIb and GpIIIa, outdated human platelets were extracted with 1% Triton X-100 in 0.05 mol/L Tris buffer, pH 7.4, containing 1 mmol/L $CaCl_2$.[13] Affinity chromatography was then performed with the GpIIb/GpIIIa complex-specific monoclonal antibody A_2A_9 immobilized on Sepharose.[14] Retained GpIIb-GpIIIa complexes were eluted with 0.05 mol/L diethylamine, pH 11.5, and precipitated with 6 vol of cold acetone.[14] The eluted GpIIb and GpIIIa were then separated by 0.1% sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gel electrophoresis performed in the absence of a reducing agent.[15] Regions of the gels containing GpIIb and GpIIIa were excised, and the glycoproteins were removed by electroelution. Prior to immunizing rabbits, GpIIb and GpIIIa were reduced with 0.2 mol/L dithiothreitol and alkylated with 0.25 mol/L iodoacetamide. Anti-GpIIb and anti-GpIIIa IgG were prepared from rabbit immune serum by affinity chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Prior to use, the IgG were incubated with thrombasthenic platelets and with either purified GpIIb or GpIIIa fixed to Immulon 2 plates (Dynatech Laboratories, Alexandria, VA).

Immunoblotting with anti-GpIIb and anti-GpIIIa IgG. HEL cells (a gift from Dr Thalia Papayannopoulou, University of Washington, Seattle) and K562 cells were grown in RPMI 1640 medium (Flow Laboratories, McLean, VA) containing 10% fetal calf serum. Log growth phase cells were seeded at 0.5 × 10^6/mL. For some experiments, K562 cells were grown in the presence of 10^{-8} mol/L phorbol-12-myristate-13-acetate (PMA) (Sigma Chemical Co, St Louis) because this phorbol ester was reported to induce the expression of platelet antigens by this cell line.[12] Cells were extracted with 0.5% Triton X-100 in 0.05 mol/L Tris buffer, pH 7.4, containing 0.15 mol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L *N*-carbobenzoxy-L-glutamyl-L-tyrosine (buffer A). Aliquots of the extracts containing 150 to 300 μg protein were then electrophoresed on 0.1% SDS-7.5% polyacrylamide slab gels. Disulfide reduction was accomplished with 10% 2-mercaptoethanol. Following electrophoresis, separated proteins were transferred to nitrocelluose paper (Schleicher & Schuell, Inc, Keene, NH). Immunoblotting was performed using a 1/20 dilution of either rabbit anti-GpIIb or anti-GpIIIa IgG and ^{125}I-labeled staphylococcal protein A (Boehringer Mannheim, Indianapolis) as previously described.[16] The labeled immunoblots were dried and exposed to Kodak X-Omat AR photographic film for 24 to 96 hours.

Immunoprecipitation of GpIIb and GpIIIa from HEL and K562 cells. HEL cells, K562 cells, and human platelets were labeled with ^{125}I using lactoperoxidase/H_2O_2 and were extracted with 0.5%

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Triton X-100 in buffer A. Fixed staphylococci (Pansorbin, Calbiochem-Behring Corp, La Jolla, CA) were used to collect immune complexes and were prepared by heating at 100°C for ten minutes in 2 mMol/L Tris buffer, pH 7.5 containing 3% SDS and 10% 2-mercaptoethanol and washing five times with 2 mmol/L Tris buffer, pH 7.5, containing 0.1% SDS and 0.1% Triton X-100.[17] Cell solutions were cleared by incubating 100-μL aliquots with 2 μL of nonimmune rabbit serum for 15 minutes at 4°C followed by an incubation with 10 μL of a 10% suspension of staphylococci for 15 minutes at 4°C. Ten to 20 μg of either anti-GpIIb or anti-GpIIIa IgG were then added to the supernatants and the incubations continued at 4°C for 16 hours. Immune complexes were collected by adding 100 μL of a 10% suspension of staphylococci for 1 hour at 4°C. The staphylococci were then washed five times with 2 mmol/L Tris buffer, pH 7.5, containing 0.15 mol/L NaCl, 0.1% SDS, and 0.1% Triton X-100 and were heated at 100°C for five minutes in 0.01 mol/L Tris-HCl buffer, pH 6.8, containing 3% SDS. Immune complexes were electrophoresed on unreduced 0.1% SDS–7.5% polyacrylamide slab gels. The gels were dried and exposed to Kodak X-Omat AR film for one to three days.

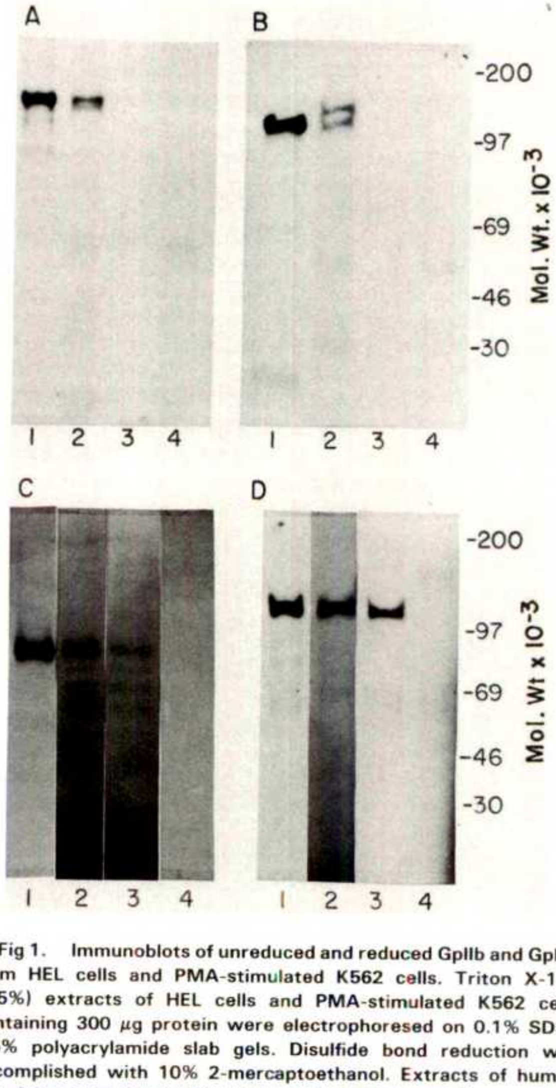

Fig 1. Immunoblots of unreduced and reduced GpIIb and GpIIIa from HEL cells and PMA-stimulated K562 cells. Triton X-100 (0.5%) extracts of HEL cells and PMA-stimulated K562 cells containing 300 μg protein were electrophoresed on 0.1% SDS–7.5% polyacrylamide slab gels. Disulfide bond reduction was accomplished with 10% 2-mercaptoethanol. Extracts of human platelets were prepared in the same manner as positive controls. The separated proteins were electrophoretically transferred from the gels to nitrocellulose paper and were examined by immunoblotting by using rabbit anti-GpIIb and anti-GpIIIa IgG and ^{125}I-labeled staphylococcal protein A. Autoradiograms of the dried blots are shown in the figure. Molecular weights were calculated from the mobility of standard proteins. (A, B) Immunoblots using anti-GpIIb IgG. (C, D) Immunoblots using anti-GpIIIa IgG. (A, C) Immunoblots of unreduced gels. (B, D) Immunoblots of reduced gels: 1, platelets; 2, HEL cells; 3, K562 cells grown in the presence of 10^{-8} mol/L PMA for 48 hours; 4, unstimulated K562 cells.

Detection of platelet GpIIb and GpIIIa antigens on the surface of intact HEL and K562 cells. To detect platelet-specific GpIIb and GpIIIa antigens on the surface of intact HEL and K562 cells, 0.35 × 10^6 cells were incubated with dilutions of a panel of antihuman platelet GpIIb, GpIIIa, and GpIIb-IIIa murine monoclonal antibodies for 30 minutes at 4°C in a buffer consisting of Dulbecco's phosphate-buffered saline (GIBCO, Grand Island, NY) with 0.02% sodium azide and 0.2% bovine serum albumin. After a second incubation with a 1/40 dilution of affinity-purified fluorescein isothiocyanate–conjugated goat (Fab')$_2$ antimurine immunogobulin (Tago Diagnostics, Burlingame, CA) for 30 minutes at 4°C, the cells were examined in a fluorescent cell analyzer (Spectrum III, Ortho Diagnostics, Westwood, MA). Antibody binding was considered positive if the number of fluorescent cells exceeded the number seen with the negative control antibody P3 × 63. The monoclonal anti-GpIIb antibody Tab was a gift from Dr Rodger McEver.[18] The monoclonal antibody B1B5 was produced by immunizing mice with purified human platelet GpIIb. It binds only to platelet GpIIb on immunoblots of human platelet membranes.

In vitro translation of GpIIb and GpIIIa. GpIIb and GpIIIa precursors were translated in vitro using RNA purified from HEL and K562 cells by the method of Deeley et al.[19] For some experiments mRNA was isolated from the total cellular RNA by chromatography on oligo-dT cellulose (Bethesda Research Laboratories, Gaithersburg, MD). RNA translations were performed at 37°C for 60 minutes in the presence of dithiothreitol by using a rabbit reticulocyte lysate in vitro translation system (New England Nuclear, Boston). Translation of HEL RNA was performed in the presence of [^{35}S]methionine (New England Nuclear). GpIIb and GpIIIa precursors were immunoprecipitated from the translation mixture with polyclonal anti-GpIIb or anti-GPIIa IgG and identified by SDS–polyacrylamide gel electrophoresis performed under reducing conditions. The specificity of the immunoprecipitations was tested by preincubating the IgG with 5 to 10 μg of either purified platelet GpIIb or GpIIIa. Following electrophoresis, the gels were prepared for fluorography (Autofluor, National Diagnostics, Somerville, NJ), dried, and exposed to Kodak X-Omat AR film at −70°C for one to three days. RNA purified from K562 cells was translated in vitro in the presence of unlabeled methionine. The translation products were dissolved in SDS and examined by immunoblotting. Monospecific polyclonal rabbit anti-GpIIb and anti-GpIIIa antisera and ^{125}I-labeled staphylococcal protein A were used for these blots.

RESULTS

Characterization of HEL and K562 GpIIb and GpIIIa. To detect HEL GpIIb and GpIIIa, cell extracts were prepared with Triton X-100, electrophoresed on SDS–polyacrylamide gels under nonreducing and reducing conditions, and examined by immunoblotting with polyclonal anti-GpIIb and anti-GpIIIa IgG (Fig 1). We found that HEL cells contained polypeptides reacting with both anti-GpIIb and anti-GpIIIa IgG. Under nonreducing conditions, anti-GpIIIa IgG reacted with a 95,000-M_r polypeptide. Following reduction, the apparent M_r of this polypeptide increased to 116,000. This increase in apparent M_r following reduction is similar to the behavior of platelet GpIIIa upon reduction and suggests that HEL GpIIIa, like platelet GpIIIa, is a single-chain polypeptide with multiple intrachain disulfide bonds.[7] Anti-GpIIb IgG under nonreducing conditions reacted with a 140,000-M_r polypeptide. However, under reducing conditions, it reacted with two polypeptides, the 140,000-M_r polypeptide and a second polypeptide with an M_r of 125,000. Platelet GpIIb is a disulfide-linked two-chain molecule containing a 125,000-M_r α chain and a 23,000 M_r β chain.[7] Bray et al recently reported that HEL GpIIb is synthesized as a single-chain precursor that is processed posttranslation into α and β chains.[20] The M_r of the polypeptides recognized by our anti-GpIIb IgG correspond to those of platelet GpIIb and GpIIbα, respectively. This suggests that HEL cells contain two pools of GpIIb molecules, one containing disulfide-linked α and β chains and a second that did not undergo cleavage into α and β chains.

We also performed immunoblotting with extracts of unstimulated and PMA-stimulated K562 cells (Fig 1). Anti-GpIIIa IgG did not react with any polypeptides on the immunoblots of the unstimulated cells but did react with a 95,000-M_r polypeptide after the cells had been incubated with 10^{-8} mol/L PMA for at least 12 hours. Furthermore, like HEL and platelet GpIIIa, the molecular weight of this polypeptide increased to 116,000 following disulfide reduction. However, in contrast to HEL cells and platelets, anti-GpIIb IgG did not react with any polypeptides on immunoblots of extracts of either unstimulated K562 cells or of K562 cells stimulated with PMA for as long as 72 hours.

To determine whether HEL GpIIb and GpIIIa and K562 exist in heterodimer complexes, we performed immunoprecipitations using Triton X-100 extracts of ^{125}I-labeled cells and the polyclonal anti-GpIIb and anti-GpIIIa IgG. Immunoprecipitations also were performed with ^{125}I-labeled human platelets. As seen in Fig 2, anti-GpIIb and anti-GpIIIa IgG each immunoprecipitated both components of the GpIIb-GpIIIa heterodimer from the platelet extracts.[13] Similarly, anti-GpIIb IgG immunoprecipitated both GpIIb and GpIIIa from the HEL cell extracts. This result indicates that at least a portion of the HEL GpIIb and GpIIIa exists in heterodimer complexes. Due to partial reduction of the GpIIb during the immunoprecipitation, a polypeptide corresponding to GpIIbα was also present in this immunoprecipitate. Anti-GpIIIa IgG also immunoprecipitated GpIIb and GpIIIa from the dissolved HEL cells. However, the predominant polypeptide in this immunoprecipitate was GpIIIa. The reason for the difference in the composition of the HEL immunoprecipitates depending on whether anti-GpIIb and anti-GpIIIa IgG was used is not clear because the immunoprecipitations were performed under identical conditions but a similar difference in the composition of the immunoprecipitates was seen in the platelet controls.

As we expected from the K562 immunoblots, anti-GpIIIa immunoprecipitated a GpIIIa analogue from extracts of PMA-stimulated K562 cells but not from extracts of unstimulated cells. However, we did not detect another polypeptide complexed with GpIIIa in these immunoprecipitates, nor was a GpIIb analogue present in immunoprecipitates using anti-GpIIb IgG. These data confirm that PMA-stimulated K562 cells express a GpIIIa-like protein and do not express a protein antigenically similar to platelet GpIIb. They also suggest that GpIIIa does not exist in a complex in PMA-stimulated K562 cells.

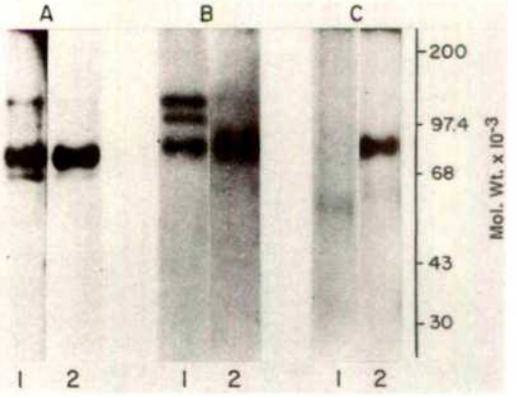

Fig 2. Identification of GpIIb and GpIIIa antigens from HEL and K562 cells by immunoprecipitation. Immunoprecipitations were performed by using ^{125}I-labeled HEL cells, K562 cells, and human platelets dissolved with 0.5% Triton X-100 in a buffer containing 0.05 mol/L Tris, 0.15 mol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L *N*-carbobenzoxy-L-glutamyl-L-tyrosine, pH 7.4, and either monospecific rabbit anti-GpIIb or anti-GpIIIa IgG. Immune complexes were collected with fixed staphylococci and were examined on unreduced 0.1% SDS–7.5% polyacrylamide slab gels. Following electrophoresis, the gels were dried and exposed to Kodak X-omat AR film for one to three days. Molecular weights were calculated from the mobility of concurrently run standard proteins. (A) Human platelets. (B) HEL cells. (C) K562 cells grown in the presence of 10^{-8} mmol/L PMA for 48 hours: 1, immunoprecipitation using anti-GpIIb IgG; 2, immunoprecipitation using anti-GpIIIa IgG.

Last, to compare the antigenic reactivity of HEL and K562 GpIIb and GpIIIa antigens with their platelet counterparts, we incubated cells from each line with a panel of murine antihuman platelet GpIIb and GpIIIa monoclonal antibodies. Although these antibodies react readily with platelets, they do not react with lymphocytes, monocytes, or neutrophils (data not shown). The cells were then incubated with fluorescein-conjugated antimurine IgG F(ab')$_2$ and examined with a fluorescent cell analyzer. The majority of HEL cells exhibited fluorescence after incubation with monoclonal antibodies against human platelet GpIIb, GpIIIa, and the GpIIb/IIIa complex (Table 1). In contrast, K562 cells only exhibited background fluorescence when incubated with the same antibodies. However, after these cells were exposed to 10^{-8} mol/L PMA, the majority exhibited fluorescence after incubation with the anti-GpIIIa monoclonal antibody A6. A small increment in fluorescence was also seen when the PMA-stimulated K562 cells were incubated with monoclonals against GpIIb and the GpIIb/IIIa complex. However, this increment was the result of an increase in background fluorescence that followed cell stimulation with PMA, consistent with our inability to detect GpIIb synthesis in K562 cells by immunoblotting or immunoprecipitation.

In vitro synthesis of GpIIb and GpIIIa by using RNA from HEL cells. Purified HEL RNA was translated in vitro by using a rabbit reticulocyte lysate translation system in the presence of [^{35}S]methionine. Newly synthesized poly-

Table 1. Binding of Antihuman Platelet Monoclonal Antibodies to Human Platelets and to the Human Leukemic Cell Lines HEL and K562

Antibody	Specificity	Percent Binding to Platelets	Percent Binding to HEL Cells [6]	Percent Binding to K562 Cells [4] (no PMA/PMA)
P3 × 63	Nonimmune	0.1	3	2/4
A6	IIIa	97.1	57	4/68
A2A9	IIb-IIIa	75.3	61	5/10
B1B5	IIb	81.7	42	2/11
Tab	IIb	—	—	4/6

K562 cells were grown for 48 hours in the presence or absence of 10^{-8} mol/L PMA. These cells and HEL cells were incubated for 30 minutes at 4 °C with a panel of antihuman platelet monoclonal antibodies as described under Methods. The cells were then washed and incubated for 30 minutes at 4 °C with a 1/40 dilution of affinity-purified fluorescein isothiocyanate-conjugated goat $(Fab')_2$ antimurine IgG before they were studied in a fluorescence cell analyzer. Fluorescence thresholds for positivity were adjusted by using the antibody P3x63 as a negative control. Percent binding indicates the percentage of the analyzed cells whose fluorescence was greater than the threshold. The number in brackets indicates the number of experiments. The data are mean values.

peptides containing GpIIb and GpIIIa antigens were identified by immunoprecipitation using monospecific rabbit IgG. Following the translation of 6.2 μg of HEL RNA, anti-GpIIb IgG immunoprecipitated a single ^{35}S-labeled polypeptide with an M_r of approximately 110,000 (Fig 3). The immunoprecipitation was specific for a GpIIb precursor because it was inhibited by preincubating the IgG with purified platelet GpIIb. Likewise, following translation of 25 μg of HEL RNA, anti-GpIIIa IgG immunoprecipitated a labeled polypeptide with an M_r of approximately 92,000. Preincubation of the anti-GpIIIa IgG with purified platelet GpIIIa also inhibited this immunoprecipitation. Thus, HEL cell RNA directed the synthesis of distinct precursors for GpIIb and GpIIIa. Because no processing of polypeptides occurs in our in vitro translation system and because in eukaryotes each species of cellular mRNA codes for a single polypeptide, our results indicate that the synthesis of GpIIb and GpIIIa is directed by separate mRNAs.

In vitro synthesis of GpIIIa by using RNA from K562 cells. The K562 RNA in vitro translation products were examined by immunoblotting by using rabbit polyclonal anti-GpIIb and anti-GpIIIa antisera and ^{125}I-labeled staphylococcal protein A (Fig 4). Anti-GpIIIa antiserum identified a single 92,000-M_r polypeptide whose synthesis was directed by RNA from PMA-stimulated cells. The mobility of this polypeptide was the same as the HEL polypeptide immunoprecipitated with anti-GpIIIa IgG. A faint band with the same mobility was also seen in the translation directed by RNA from non–PMA-stimulated cells, but no bands were present when water or globin mRNA was substituted for K562 RNA. Likewise, no polypeptides reacting with anti-GpIIb antiserum were detected in these translations (data not shown). Thus, PMA-stimulated K562 cells express a gene for GpIIIa. If the gene for GpIIb is also expressed, then its level of expression is below our limits of detection.

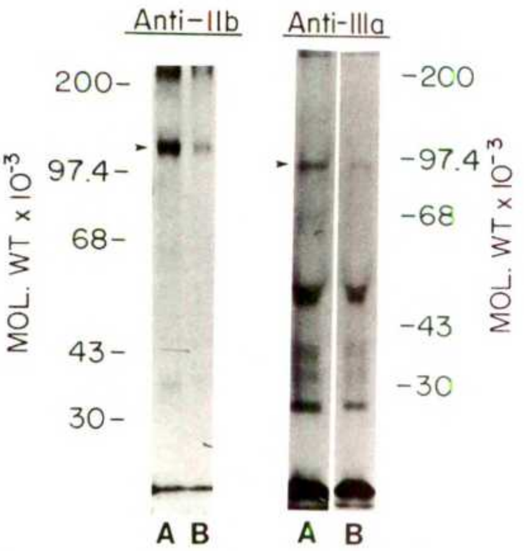

Fig 3. In vitro translation of GpIIb and GpIIIa using RNA from HEL cells. HEL cell RNA was purified and translated in vitro in the presence of [^{35}S]methionine by using a rabbit reticulocyte lysate translation system. Newly synthesized GpIIb and GpIIIa were identified by immunoprecipitation by using rabbit anti-IIb and anti-IIIa IgG. The specificity of the immunoprecipitations was tested by preincubating the IgG with 5 to 10 μg of either purified platelet GpIIb or GpIIIa. In the experiment shown, translation of 6.2 μg of total cell RNA was required to detect GpIIb, whereas the detection of GpIIIa required the translation of 25 μg of RNA. This figure is an autofluorogram of a 0.1% SDS–7.5% polyacrylamide gel of the immunoprecipitates. (A) Immunoprecipitation in the absence of purified platelet protein. (B) Immunoprecipitation in the presence of purified platelet protein.

DISCUSSION

The platelet membrane glycoproteins IIb and IIIa form a heterodimer complex that is of critical important for normal platelet function because it is the platelet surface structure that mediates platelet aggregation.[14] Evidence is also accumulating that platelet GpIIb and GpIIIa are members of a family of adhesive protein receptors. Proteins antigenically and structurally related to platelet GpIIb and GpIIIa have been found on endothelial cells,[21,22] and complexes similar to GpIIb-IIIa are present on myeloid and lymphoid cells.[23] For example, the OKM1 antigen found on myeloid cells and the LFA-1 antigen found on lymphoid and myeloid cells are both noncovalent heterodimers containing a common 94,000-M_r glycoprotein complexed with either a 155,000- or a 177,000-M_r glycoprotein.[23] Both OKM1 and LFA-1 are involved in the adherence of effector cells to target cells.[24,25] Recently, Cosgrove et al isolated a single clone from a human genomic library that when expressed produced antigens found on the large subunits of OKM1 and LFA-1 and on GpIIb.[26] Although the large subunits of OKM1, LFA-1, and GpIIb-IIIa are distinct glycoproteins, these data suggest that the proteins contain common amino acid sequences that have been conserved for cellular adhesive functions. Burns et al have directly implicated the GpIIb-IIIa complex in leukocyte adhesion.[27] They produced a monoclonal antibody against lymphocytes that not only modified leukocyte function but also inhibited platelet aggregation and immunopre-

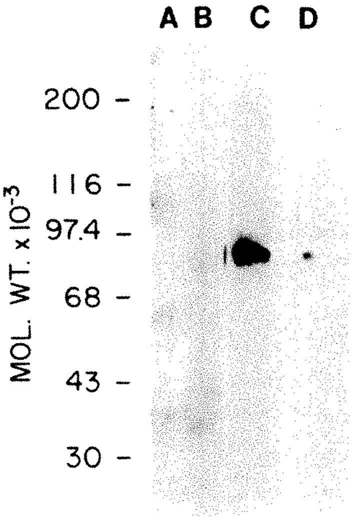

Fig 4. In vitro translation of GpIIIa using RNA from K562 cells. RNA was isolated from K562 cells and translated in vitro as described in Fig 3 except that the translation was performed with unlabeled methionine. The translation products were subjected to SDS–polyacrylamide gel electrophoresis as described in Fig 3 and examined by immunoblotting as described in Fig 1. Monospecific rabbit anti-GpIIIa IgG and ^{125}I-labeled staphylococcal protein A were used in these blots. (A, B) control translations in which water and globin mRNA were substituted for K562 RNA, respectively. (C) Translation directed by RNA from K562 cells exposed to PMA for 48 hours. (D) Translation directed by RNA from K562 cells grown for 48 hours in the absence of PMA.

cipitated the platelet GpIIb-IIIa complex. On the other hand, it is unlikely that there is complete identity between platelet GpIIb and GpIIIa and their leukocyte counterparts because none of our panel of anti-GpIIb and anti-GpIIIa monoclonal antibodies reacts with lymphocytes, monocytes, or neutrophils (data not shown).

Reports that the function of several adhesive protein receptors can be inhibited by peptides containing the amino acid sequence Arg-Gly-Asp-Ser strengthens the concept for a family of adhesion protein receptors.[28] These receptors include the platelet GpIIb-IIIa complex[29-31] and the vitronectin and fibronectin receptors found on fibroblasts, MG-63 osteosarcoma cells, and human placenta.[32,33] Moreover, the vitronectin receptor is a heterodimer whose subunits behave like GpIIb-IIIa on unreduced and reduced SDS-gels.[34] In contrast, the mammalian fibronectin receptor appears as a single 140,000-M_r glycoprotein on reduced gels and as a 140,000/120,000-M_r heterodimer on unreduced gels.[32] In addition, although platelet GpIIb-IIIa appears to be capable of interacting with fibrinogen, fibronectin, and vitronectin, the vitronectin and fibronectin receptors interact solely with their respective ligands, which suggests that important structural difference may exist among the members of the adhesive protein receptor family.[34]

In order to examine the possibility of homology between platelet GpIIb-IIIa and adhesive protein receptors on other cells and to determine whether the synthesis of GpIIb and GpIIIa is under coordinate control, we have searched for cells containing GpIIb and GpIIIa other than megakaryocytes. Although megakaryocytes would seem to be the appropriate cells for our studies, their paucity in bone marrow specimens precludes their use. In confirmation of previous reports[11,12] we found the antigens for several antihuman platelet GpIIb and GpIIIa monoclonal antibodies on HEL cells and an antigen for platelet GpIIIa, but not for platelet GpIIb, on PMA-treated K562 cells. In addition, using polyclonal antihuman platelet GpIIb and GpIIIa IgG, we found that the structures containing these antigens were similar in molecular weight and subunit composition to the corresponding platelet proteins. Thus, HEL cells and PMA-treated K562 cells appear to represent readily available resources for study of GpIIb and GpIIIa synthesis.

As a first step in our studies, we isolated RNA from the HEL cells and from unstimulated and PMA-stimulated K562 cells and translated the RNA in vitro. HEL RNA directed the synthesis of distinct precursors for both GpIIb and GpIIIa, which indicates that the synthesis of each is directed by separate species of mRNA. Thus, GpIIb and GpIIIa do not arise from the posttranslation cleavage of a common precursor. Bray et al have also reported that GpIIb and GpIIIa synthesis by HEL cells is directed by separate mRNAs.[20] In addition, their results suggest that GpIIb and GpIIIa are processed before delivery to the plasma membrane. Confirming their results, we found that the apparent molecular weights of the HEL GpIIb and GpIIIa precursors translated in vitro are consistent with the expected molecular weights of the reduced, nonglycosylated platelet species. Human platelet GPIIb and GPIIIa contain 14% and 16% carbohydrate, respectively.[6] Thus, the estimated molecular weight of nonglycosylated platelet GPIIb containing both α and β chains is 116,000,[7] close to the value for the HEL GPIIb precursor we translated in vitro. A similar estimate for reduced, nonglycosylated platelet GpIIIa is 91,000, again close to the molecular weight of the HEL GpIIIa precursor.

RNA from PMA-stimulated K562 cells directed the synthesis for a GpIIIa precursor that was very similar in size and antigenicity to the HEL GpIIIa precursor. On the other hand, in spite of data that most adhesive protein receptors exist as heterodimers, we did not detect a protein containing GpIIb antigens on PMA-stimulated K562 cells, nor could we translate a GpIIb precursor in vitro using K562 RNA. It is possible that K562 cells produce a protein antigenically unrelated to GpIIb that forms a heterodimer with GpIIIa. However, if such a protein is present it would seem to be substantially different from both platelet and HEL GpIIb.

The simplest explanation for the presence of separate mRNAs for GpIIb and GpIIIa in HEL cells is that each glycoprotein is coded by a separate gene. The dissociation of GpIIb from GpIIIa expression in PMA-stimulated K562 cells is consistent with this explanation. Consequently, it may be necessary to postulate either linked abnormalities in the transcription of the genes for GpIIb and GpIIIa or linked abnormalities in the postsynthetic processing of the GpIIb and GpIIIa precursors to explain the glycoprotein abnormalities in thrombasthenia. Alternative splicing of a single immature mRNA[35] or the presence of several initiation codons within a single cellular mRNA could explain our results and the observations in thrombasthenia. However, the absence of any structural or antigenic relationship between

GpIIb and GpIIIa makes the former possibility unlikely, and the latter situation has only been rarely observed in eukaryotes.[36] Because GpIIIa seems to exist in the K562 cell membrane with little or no GpIIb, it appears that GpIIIa synthesis does not require concominant synthesis of GpIIb, and it seems unlikely that an abnormality in GpIIb synthesis or processing alone could account for thrombasthenia. Our results do not preclude a primary abnormality in GpIIIa synthesis as the explanation for thrombasthenia. However, a thrombasthenic whose platelets have a GpIIb-IIIa pattern similar to that of PMA-stimulated K562 cells has not been described. Direct examination of the genes coding for GpIIb and GpIIIa will be necessary to explain the protein abnormalities in thrombasthenia.

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Immunochemical Characterization of Fibrinogen, Fibrin I, and Fibrin II in Human Thrombi and Atherosclerotic Lesions

By Alessandra Bini, John Fenoglio Jr, Joan Sobel, John Owen, Marcela Fejgl, and Karen L. Kaplan

Arterial thrombi and atherosclerotic lesions were analyzed immunochemically and examined histologically. The extent of in vivo proteolytic cleavage of the amino-terminal end of fibrinogen by thrombin and plasmin was determined and quantitated by specific radioimmunoassays. The samples were treated with cyanogen bromide (CNBr), and the total amount of fibrinogen and fibrin-derived protein was determined as NDSK, the NH_2-terminal disulfide knot of fibrinogen. Thrombin-releasable fibrinopeptides A and B were used to quantitate fibrinogen and fibrin I. Previous plasmin cleavage of the $B\beta$ chain was inferred from the amount of $B\beta$ 1-42 and $B\beta$15-42 in undigested NDSK. The results obtained in both acute and organized thrombi indicate that ~60% of the total protein (as determined by amino acid analysis) was fibrinogen-derived and that 70% to 80% of the fibrinogen-derived material was fibrin II. These findings support the hypothesis that fibrin II as distinct from fibrin I is the predominant component in a thrombus. In samples from normal and atherosclerotic aortas, fibrinogen-derived protein comprised <10% of the total protein. Samples from grossly normal aortas contained only fibrinogen and fibrin I. Fibrinogen concentration decreased and fibrin II concentration increased with increasing severity of the lesions, suggesting that increased fibrin II formation is associated with progression of atheromas.

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SEVERAL in vitro studies have defined the biochemical structure of fibrin in vitro.[1-4] Little information is available on the composition of human thrombi and atherosclerotic plaques with respect to the content of fibrinogen and resulting fibrin products.

Previous studies on thrombi recovered from patients have been devoted to the carboxy-terminal end of the fibrinogen molecule and have shown fibrin with a high degree of α and γ chain crosslinking.[5,6] Recently, Francis and colleagues[7] demonstrated that the degree of fibrin degradation in thrombi increases progressively with the age of the thrombus and it is more prominent in the deeper layers of chronic thrombi.

The association between thrombus formation and atherosclerotic lesions has long been recognized.[8,9] The presence of fibrinogen and fibrin-related antigens in normal aortas and atherosclerotic plaques has been shown by immunofluorescence and electron microscopy by several investigators.[10-12] Smith and colleagues[13] documented that fibrinogen and fibrin in soft gelatinous human aortic intimal thickenings and cholesterol-rich advanced plaques were, respectively, four and ten times more concentrated than in normal vessels.

Shainoff and Page[14] reported that atherosclerotic plaques contained crosslinked fibrinogen, based on their estimates of fibrinopeptide content and on insolubility in 5 mol/L of urea. In normal aortas and in aortas with atherosclerotic lesions, York and Benjamin[15] found a correlation between the content of fragment D antigen and the severity of the atherosclerotic lesion. No correlation was found with the fibrinogen content of plaques.

Nossel postulated that thrombi should consist mainly of fibrin II*[16] and, in a study of postcraniotomy venous thrombosis,[17] the patients who developed venous thrombosis showed evidence of an imbalance of thrombin and plasmin action that would favor fibrin II formation.

We therefore studied the biochemical composition of the amino-terminal ends of fibrinogen and fibrin in thrombi and atherosclerotic lesions with respect to proteolysis by thrombin and plasmin. Using specific radioimmunoassays, we determined the relative amounts of fibrinogen, fibrin I, fibrin II, and fragment X,[18-22] and compared the data obtained on thrombi with results in normal and atherosclerotic arterial intima.

MATERIALS AND METHODS

Preparation of fibrin in vitro. Platelet-poor plasma was prepared from blood collected into 1/10 vol of an anticoagulant solution consisting of 3.8% trisodium citrate, 100 KIU/mL of aprotinin (Trasylol, Mobay Chemical, FBA, New York), 10 mg/mL soybean trypsin inhibitor (Worthington Biochemical, Freehold, NJ), with or without 80 U/mL of heparin (Heparin sodium, Hynson Westcott and Dunning, Baltimore), and separated by centrifugation for 20 minutes at 1,700 g at 4°C in a Sorvall RC-2 centrifuge (Newton, CN).

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*Definition of terms: fibrin I, formed from fibrinogen by cleavage of $A\alpha$16-17; fibrin II, formed from fibrinogen by cleavage of both $A\alpha$16-17 and $B\beta$14-15; NDSK, amino terminal CNBr fragment of fibrinogen; fragment X, formed by plasmin cleavage of the $B\beta$42-43 bond and α-chain bonds of fibrinogen, fibrin I, or fibrin II (includes fragments Y and E as calculated in this study).

Fibrinogen concentration was measured in citrated plasma as described by Ellis and Stransky[23] and by the clot weight method.[24] Plasma obtained from the heparin-containing anticoagulant mixture was clotted overnight at 37°C with batroxobin 1 mg/mL of plasma (Reptilase-R, Abbott Laboratories, North Chicago). Crosslinked fibrin was prepared by adding 1.0 National Institutes of Health (NIH) U of human α-thrombin (3.7×10^3 NIH U/mg) (prepared by Dr J. Fenton and kindly given to us by Dr G. Murano, Bethesda, MD) and 10 μL of a 2.5 mol/L of calcium chloride solution per milliliter of citrated plasma (final concentration 0.025 mol/L of $CaCl_2$), followed by overnight incubation at 37°C. The clots were removed by winding them on a wooden stick; they were rinsed three times for 15 minutes in distilled water, cut longitudinally with a scalpel, stirred for 1 hour in 70% vol/vol of formic acid, and incubated with 300-fold molar excess cyanogen bromide (CNBr),[20-23] for 20 to 24 hours with continuous stirring. The CNBr-treated clots were then diluted 1:11 with distilled water, lyophilized twice, resuspended in 10% NH_4OH (vol/vol) containing 0.01% Tween 20 (vol/vol), and the pH was adjusted to 8 to 8.5 with HCl. Samples were stored at −70°C until assayed.

Sample collection. Thrombi and tissue samples were collected at surgery and at autopsy under a protocol approved by the Institutional Review Board of the Columbia Presbyterian Medical Center. Specimens collected at surgery were placed, immediately on excision, into sterile 50-mL tubes filled with 0.1 mol/L of NaCl, 0.05 mol/L of Tris pH 7.4 containing 100 KIU/mL of aprotinin and 1.0 mmol/L of benzamidine (Benzamidine hydrochloride hydrate, Aldrich Chemical, Milwaukee). They were rinsed in three changes of the same buffer within 2 hours of resection. Samples collected at autopsy 6 to 24 hours postmortem were rinsed carefully in the same buffer. Aortas were incised with a scalpel, and the intima was directly separated from the media and the adventitia. The samples were then divided into grossly homogenous parts, and areas of atherosclerotic aortas were collected with paired normal adjacent areas. Each specimen was individually placed in preweighed siliconized glass vials and frozen and stored at −70°C until processed. Prior to freezing, a portion of each sample was cut and fixed in 10% neutral buffered formaldehyde for histologic examination.

The specimens for histology were paraffin embedded, and 8-μm sections were cut from the blocks and stained with hematoxylin-phloxine-saffran and Masson's trichrome. The stain allows distinction between cells, nuclei, "fibrin," and collagen. The samples were classified as acute or organized thrombi or fibrous, fatty, or complicated plaques according to standard histological criteria (Table 1).

Table 1. Clinical Data of Patients From Whom Samples Were Obtained

No.	Age	Sex	Disease	Procedure	Histology of Specimen
Surgical specimens					
1	55	M	Atherosclerosis, thrombosis left popliteal artery	Endarterectomy	Acute thrombus
2	73	M	R superior	Embolectomy	Acute thromboembolus from atheromatous plaque in abdominal aortic aneurysm
3	84	M	R common iliac artery aneurysm	Repair aneurysm	A: Complicated atherosclerotic plaque B: Complicated atherosclerotic plaque
8	74	M	Abdominal aortic aneurysm	Aneurysmectomy	Organizing thrombus
9	88	M	Abdominal aortic aneurysm	Aneurysmectomy	A: Organized thrombus B: Acute thrombus C: Organizing thrombus
11	93	F	Peripheral vascular disease	Popliteal endarterectomy	A: Fatty atherosclerotic plaque B: Acute thombus
12	52	M	Atherosclerosis, abdominal aneurysm	Repair aneurysm	Organized thrombus
13	62	M	R carotid stenosis	Endoarterectomy	Complicated atherosclerotic plaque
14	62	M	Thrombosed A-V fistula	Thrombectomy	Acute thrombus
15	75	M	Abdominal aortic aneurysm	Aneurysmectomy	A: Organized thrombus B: Organized thrombus
16	49	M	Mitral stenosis	Mitral valve commissurotomy	A: Acute thrombus
18	53	M	Left ventricular aneurism with mural thrombus formation	Aneurysmectomy	Organized thrombus
Autopsy specimens					
4	60	F	Myocardial infarction		A: Fibrous atherosclerotic plaque B: Normal aorta C: Fatty atherosclerotic plaque
5	64	F	Myocardial infarction		A: Fatty atherosclerotic plaque B: Fibrous atherosclerotic plaque
6	82	M	Myocardial infarction		A: Normal aorta B: Fibrous atherosclerotic plaque
17	65	F	Cirrhosis, sepsis		A: Normal aorta B: Fibrous atherosclerotic plaque C: Fatty atherosclerotic plaque D: Fatty atherosclerotic plaque

Definitions: Acute thrombus, fibrin clot with discernible lines of Zahn; organizing thrombus, partial replacement of fibrin clot by granulation tissue; organized thrombus, total replacement of fibrin clot by maturing granulation tissue; fibrous plaque, focal intimal accumulation of collagen and "fibroblasts"; fatty plaque, focal intimal accumulation of "foam cells," collagen, and cholesterol crystals; complicated plaque, focal accumulation of foam cells, collagen, and cholesterol crystals extending into the media and associated with calcification.

Sample processing. The tissue samples were freeze-dried in siliconized glass vials and dry-weighed. They were then homogenized in distilled water (10 to 100 mg sample per milliliter of distilled water). If the dry weight was >100 mg, samples were initially roughly homogenized on a melting ice bath with a Polytron (Brinkmann Instruments, Westbury, NY). All samples were finely homogenized at 4°C in a glass homogenizer (Arthur H. Thomas Scientific Apparatus, Philadelphia), kindly made available by Dr S. DiMauro, Department of Neurology, Columbia University. The homogenate was then diluted 1:5 with 90% formic acid and treated with cyanogen bromide as described above. The amount of CNBr added to the tissue samples was five times the dry weight of the sample. Amino acid analysis performed in a preliminary experiment demonstrated that ~50% of the dry weight was protein. This amount of CNBr corresponds to the 300-fold molar excess used for CNBr digestion of fibrin prepared in vitro.

The precipitates present in two samples were extracted a second time with formic acid and CNBr to determine whether residual NDSK antigen was present. The results are shown in Table 2. NDSK antigen was assayed on the initial extract and on the extract from the repeated CNBr treatment of the precipitate that remained after the first extraction. The recovery of fibrinogen-related material in the first extract was 95% from the intima sample and >99% from the organized thrombus. Therefore, all samples in which a precipitate was present after the first extraction were centrifuged at 12,300 g for 30 minutes, and assays for fibrinogen-related antigens were performed on the supernate.

Radioimmunoassays. NDSK, native fibrinopeptide A (FPA) and fibrinopeptide B (FPB), and Bβ 1-42 were prepared as previously described.[2,29-31] Tyrosinated FPA was from Schwarz/Mann, Becton Dickinson, Orangeburg, NY. Tyrosinated FPB was prepared by G. Wilner.[2] Antiserum to NDSK was lot QT8[29]; to FPA it was lot R2,[30,32,33] to FPB it was lot R28,[34] and to Bβ1-42 it was lot 142-3.[31] The Bβ15-42 assay kit was purchased from IMCO (MICO USA, New York).

All fragments were labeled by the method of Hunter and Greenwood.[35] Sodium iodide -^{125}I (1 to 2 mCi) was obtained from New England Nuclear (Boston). After iodination, NDSK was passed over Sephadex G-100, and the other peptides and fragments were passed over Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, NJ). NDSK was assayed with a slightly different technique from that previously described.[29] The test was performed in 12 × 75-mm polypropylene tubes (Sarstedt, Princeton, NJ). Both tracer and anti-NDSK antibody were diluted in 0.15 mol/L of Tris-NaCl pH 8.5 containing 1% bovine serum albumin (wt/vol) (Pentex Fraction V, lot 19, Miles Laboratories, Elkhart, IN), 1/50 (vol/vol) final concentration normal rabbit serum (Rabbit Whole Serum lot 23008, Cappel Scientific, Malvern, PA) and 0.01% NaN_3 (wt/vol). After overnight incubation at 4°C, goat anti-rabbit IgG antiserum (lot 0050, Miles Scientific, Naperville, IL) was added at 1/10 dilution in Tris-NaCl containing 1% bovine serum albumin (BSA) and 2% polyethylene glycol (wt/vol) (PEG 8000, J.T. Baker Chemical, Phillipsburg, NJ). The samples were incubated for 2 hours at 4°C, and the bound antigen was separated by centrifugation, as previously described. Radioimmunoassays for FPA,[30] FPB,[2] Bβ1-42,[31] and Bβ15-42[36] were performed by our published double-antibody technique, substituting first antibody, tracer, standard, and second antibody as appropriate.[37]

The assay buffer for all of these assays is 0.15 mol/L of Tris-NaCl pH 8.0 containing 1.0% PEG 8000, heparin (0.7 U/mL), 0.01% Tween 20, and 0.01% NaN_3. The bound antigen was separated by centrifugation at 1,700 g for 20 minutes, the supernate was aspirated, and the precipitate was counted for 1 minute with an LKB 1270 Rackgamma II, (LKB Instruments, Rockville, MD).

Calculation of fibrinogen-derived material. The radioimmunoassays applied to identify and quantitate the fibrinogen NH_2-terminal antigens present in the CNBr-treated samples are shown in Table 3.

The radioimmunoassay (RIA) for NDSK detects fibrinogen and fibrin quantitatively. Experiments were made to characterize the anti-NDSK antiserum used. Comparable immunoreactivity was observed in the following preparations: NDSK, thrombin-treated NDSK,[38] plasmin-treated NDSK, reduced and alkylated NDSK, and CNBr-degraded fibrinogen derived from early (5 to 20 minutes), and late (60 minutes and overnight) plasmin digests. Non-CNBr–treated fragment E is 30 times less reactive than the other preparations.[29] The NDSK assay accurately measured all fibrinogen and fibrin-related material in the samples, however, since all samples were treated with CNBr before assay.

The RIA for fibrinopeptide A measures only the free peptide.[30,32,33] Thus, the fibrinopeptide A released by thrombin treatment of the samples provides a measure of residual fibrinogen. The FPB RIA similarly measures only the free peptide. Fibrinopeptide B released by thrombin treatment thus gives the sum of the fibrinogen and fibrin I in the samples examined.

In the Bβ1-42 RIA, the antibody used requires an intact Bβ14-15 peptide bond, and it recognizes the Bβ1-42 fragment either as a free peptide or within fibrinogen or NDSK. It is therefore a measure of both fibrinogen and fibrin I, but not of fibrin II or fragment X, which lack FPB or Bβ1-42, respectively.

The antibody used in the Bβ15-42 RIA crossreacts completely with all the Bβ15-42–containing fragments derived from CNBr treatment. Therefore, Bβ15-42 provides a measure of fibrinogen, fibrin I, and fibrin II, but not of fragment X.

The total fibrinogen-derived material and the proportions of fibrinogen, fibrin I, fibrin II, and fragment X in samples can be calculated from these data using the formulae shown in Table 4.

The molar amount of NDSK represents fibrinogen plus fibrin I, fibrin II, and fragment X. The percentage of intact fibrinogen is calculated as the difference in FPA immunoreactivity before and after thrombin treatment. Because measurement of either releasable FPB or of Bβ1-42 provides an index of fibrin I, the mean of these two

Table 2. Recovery of Fibrinogen-Related Material by CNBr Extraction

	NDSK (pmol/mL)		
Sample	First Extraction	Repeat Extraction	Recovery in First Extraction (%)
Fibrous plaque	50	2.5	95
Organized thrombus	28,180	7.0	>99

NDSK, NH_2-terminal disulfide knot of fibrinogen; CNBr, cyanogen bromide.

Table 3. Radioimmunoassays Applied to Study Proteolysis of NH_2-Terminal End of Fibrinogen by Thrombin and Plasmin

Assay for Antigen	After Secondary Digestion of CNBr Digest by	Reflects NDSK Derived From
NDSK	—	Total
Fibrinopeptide A	Thrombin	Fibrinogen
Fibrinopeptide B	Thrombin	Fibrinogen Fibrin I
Bβ 1-42	—	Fibrinogen Fibrin I
Bβ 15-42	—	Fibrinogen Fibrin I Fibrin II

Abbreviations as in Table 2.

Table 4. Summary of Calculations of Percentage of Fibrinogen, Fibrin I, and Fibrin II in NDSK

Fibrinogen = FPA*/2 NDSK† × 100
Fibrin I = [(FPB + Bβ1-42)/2 − FPA]/2 NDSK × 100
Fibrin II = (Bβ15-42 − [(FPB + Bβ1-42)/2])/2 NDSK × 100
Fragment X = (2 NDSK/2 NDSK × 100) − (Bβ15-42/2 NDSK × 100)

Abbreviations as in Table 2.

*FPA and FPB represent the total amount of fibrinopeptides measured after incubation with thrombin, from which the value obtained before thrombin treatment was subtracted.

†NDSK measured by radioimmunoassay represents 100% of the fibrinogen-related material in each sample. Because 1 mol of NDSK contains 2 mol of each of the fibrinopeptides, the amount of NDSK is multiplied by a factor of 2 in the above formulae.

measurements minus the previously calculated value for intact fibrinogen is used to calculate the percentage of fibrin I more accurately than does a measurement based only on fibrinopeptide B release from NDSK.[3,4] Fibrin II lacks both fibrinopeptides but contains the Bβ15-42 fragment. It is therefore calculated by subtracting the molar amount of Bβ1-14 containing molecules from the total Bβ15-42 immunoreactivity.

Because fragment X arises by cleavage of the Bβ42-43 bond, with loss of Bβ15-42, the percentage of fragment X in the samples can be calculated by subtracting the percentage of Bβ15-42 from the percentage of total NDSK.

Amino acid analysis. The analyses were performed with a Beckman automatic amino acid analyzer model 121 MB (Beckman Instruments, Fullerton, CA), following acid hydrolysis under a vacuum (6 N HCl for 24 hours at 110°C). All chemicals were of reagent grade and were purchased from Fisher Scientific, Springfield, NJ.

Statistical analysis. The statistical significance of variation of the content of fibrinopeptides in the thrombi and atherosclerotic plaques was tested by one-way analysis of variance. In the analysis of variance, a variance is computed from the within-group variation, and the statistical significance of the between-group variation is tested by comparing the ratio of the between-group to within-group variations to the appropriate F value in a table. The Bonferroni procedure was used to control the significance level for multiple comparisons, ie, the significance levels .05 and .01 were divided by the number of comparisons, which was three.[39]

RESULTS

Validation of methods. The amounts of each peptide expected to be present in the batroxobin and thrombin clots formed in vitro are shown in Table 5(A). In the case of the clot formed with thrombin, all FPA and FPB would be expected to be cleaved. For the batroxobin clot, only FPA would be removed and the amino terminus of the Bβ chain should be intact. Because each molecule of fibrinogen contains two Aα and Bβ chains, the expected molar amounts of the amino terminal peptides are double that of NDSK. The recovery of these peptides was >85% in each case, and was >90% for NDSK. When these data were treated as though these samples were unknowns, the calculated compositions given in Table 5(B) were obtained. These results show the expected predominance of fibrin I in the batroxobin clot and fibrin II in the thrombin clot.

Tissue samples. The samples examined and the surgical procedures from which they were obtained are shown in Table 1. The samples were classified histologically as acute or organized thrombi; fibrous, fatty, or complicated atheromatous plaques; or "normal" aortas.

Table 6 shows the fibrinopeptide content of the thrombi examined. The fibrinogen-related material determined immunologically as NDSK comprised 50% to 70% of the total protein as measured by amino acid analysis. In both acute and organized thrombi, the FPA content indicates that <2% of the NDSK was fibrinogen. The amount of releasable FPB in both groups was <5% of the NDSK, indicating that >95% of the Bβ1-14 peptide had been cleaved. Because 87% and 79% of the Bβ15-42 was present, the Bβ1-14 was cleaved by thrombin, leading to fibrin II formation, rather than by plasmin as Bβ1-42. No significant differences in the content of Bβ1-42 and Bβ15-42 or of releasable FPA and FPB were found when acute thrombi were compared with organized thrombi, indicating that they were biochemically similar at the NH_2-terminus.

Analysis of the aortic samples (Table 7) showed that <10% of the total protein was fibrinogen derived, ie, a 10- to 25-fold lower fibrinogen-related protein content than thrombi. The samples of normal aorta contained amounts of fibrinogen-related protein similar to those in the various types of plaques. In the samples of normal aorta, 79% of the fibrinogen Aα chains were intact. The amount of intact Aα-chain decreased from normal to fatty, from fatty to fibrous, and from fibrous to complicated plaques, suggesting an increase in Aα-chain proteolysis. The FPA content of complicated plaques was significantly less than that of normal aorta ($P < .01$), fatty plaques ($P < .01$), and fibrous plaques ($P < .01$). No significant differences in FPA content existed between normal aortas and either fatty or fibrous plaques or between fatty and fibrous plaques. The amount of

Table 5. Content and Composition of Fibrinogen-Related Material in In Vitro Clots

	A					B			
	Content by Radioimmunoassay					Calculated Composition			
	NDSK	FPA (μmol)	FPB	Bβ1-42	Bβ15-42	Fg	Fb I (%)	Fb II	Frag X
Batroxobin									
Observed	7.2	0.15	12.4	13.3	13.5	1.0	88	5	6
Expected	7.35	0	14.7	14.7	14.7	0	100	0	0
Thrombin									
Observed	6.5	0.13	0.13	0.40	13.2	1.0	1.8	90	8
Expected	7.35	0	0	0	14.7	0	0	100	0

Fibrinogen (Fg), Fibrin I (FbI), Fibrin II (FbII), Fragment X (Frag X), other abbreviations as in Table 2.

Table 6. Fibrinopeptide Content of Thrombi

	NDSK (%)	Antigen*			
Sample	Total Protein	FPA	FPB	Bβ 1-42	Bβ 15-42
Acute (6)	50 ± 13	1.3 ± 0.3	2.5 ± 0.7	4.4 ± 1.4	87 ± 8
Organized (6)	68 ± 5	0.6 ± 0.2	1.6 ± 0.4	2.9 ± 0.9	79 ± 11

Abbreviations as in Table 2.

*Expressed as percentage of total NDSK (mean ± SEM). No significant difference between acute and organized thrombi was found for any of the peptides.

intact Bβ-chain in the normal aortas was 91% (mean of FPB and Bβ1-42) and was not significantly different in the fatty plaques, whereas fibrous plaques showed a significant increase of Bβ1-14 cleavage as compared with both fatty plaques and normal aortas ($P < .01$). Residual FPB in the complicated plaques represented only 5% of the theoretical maximum content and was significantly less than the FPB in the other groups ($P < .01$ for each), indicating that virtually all the fibrinogen was fibrin II or fragment X. No significant differences in the content of Bβ15-42 were detected between any of the aortic samples. The mean recovery of Bβ15-42 was 73% to 92% of the theoretical amount, based on the NDSK, again indicating that the major loss of Bβ1-14 was due to thrombin action at the Bβ14-15 bond rather than to plasmin action at the Bβ42-43 bond. A summary of the data expressed as percentages of fibrinogen, fibrin I, fibrin II, and fragment X in the NDSK (calculated as in Table 4) is shown in Fig 1.

Fibrin II and fragment X together represent >95% of the total fibrinogen-related protein in both acute and organized thrombi. Acute thrombi had a slightly higher proportion of fibrin II than did organized thrombi, whereas organized thrombi had a higher proportion of fragment X. The distribution of fibrinogen-related proteins in the complicated plaques was similar to that in organized thrombi, although, as shown in Tables 6 and 7, the fibrinogen-related proteins made up a much lower percentage of the total protein in the plaques and other aortic samples than in the thrombi. Fibrous and fatty plaques contained significantly more fibrinogen, and correspondingly less fibrin II and fragment X, than did complicated plaques. The NDSK in normal aortas consisted largely of fibrinogen, with small amounts of fibrin I, fibrin II, and fragment X.

DISCUSSION

The purpose of this study was to characterize the insoluble fibrinogen-derived material in thrombi and in atherosclerotic plaques. In vitro studies have shown that when fibrinogen is clotted with thrombin, fibrinopeptides A and B are released, leading to fibrin II formation.[3,4] A thrombus is believed to contain mainly fibrin II, but this has never been directly demonstrated. To this end, we developed a method to analyze the fibrinogen-derived material in tissues. The peptides and fragments released on thrombin and plasmin proteolysis were quantitated after CNBr degradation. Application of these techniques to clots formed in vitro demonstrated both satisfactory recovery of the fibrinogen-derived material and the retention of immunoreactivity of fibrinopeptides A, B, Bβ1-42 and Bβ15-42. Thrombin clots formed in vitro with plasmin inhibitors added consisted mainly of fibrin II, as expected.

The ability to distinguish between fibrin I and fibrin II was confirmed by the data from clots formed in vitro with thrombin or batroxobin (Table 5). In those experiments, the clots contained 6% to 8% fragment X. Because the clots were formed in the presence of aprotinin and soybean trypsin inhibitor, it is unlikely that plasmin was active during that stage. One possible explanation is that this may represent the minor fraction of circulating fibrinogen which has undergone partial degradation.[40]

When these techniques were applied to the examination of pathological human thrombi (Table 6), it was found that the thrombi formed in vivo had undergone proteolysis by thrombin in a manner very similar to that of thrombin clots formed in vitro. Acute and organized thrombi did not differ significantly in fibrinopeptide content. Both contained low amounts of fibrinogen and fibrin I, whereas fibrin II comprised >70% of the fibrinogen-related protein in both groups. Moreover, 50% to 70% of the protein content of the thrombi, measured by amino acid analysis, was fibrinogen-derived in both acute and organized thrombi, confirming that the conversion of fibrinogen to fibrin II is a major factor in thrombus formation.[16]

The polyclonal antiserum to NDSK used in the present study recognizes NDSK derived by CNBr treatment of

Table 7. Fibrinopeptide Content of Aortic Samples

	NDSK (%)	Antigen*			
Sample	Total Protein	FPA	FPB	Bβ 1-42	Bβ 15-42
Normal (3)	4.6 ± 3.0	79 ± 13	73 ± 6	109 ± 4.0	88 ± 2.0
Fatty plaque (5)	1.7 ± 1.0	56 ± 14	71 ± 8	93 ± 10	92 ± 12
Fibrous plaque (3)	4.2 ± 1.8	45 ± 8	43 ± 12†	59 ± 21	73 ± 10
Complicated plaque (4)	6.1 ± 1.9	7 ± 1‡	4 ± 2‡	6 ± 1	77 ± 11

Abbreviations as in Table 2.

*Expressed as percentage of total NDSK (mean ± SEM).

†$P < .01$ as compared with fatty plaques and normal aortas.

‡$P < .01$ as compared with fibrous plaque, fatty plaque, and normal aortas.

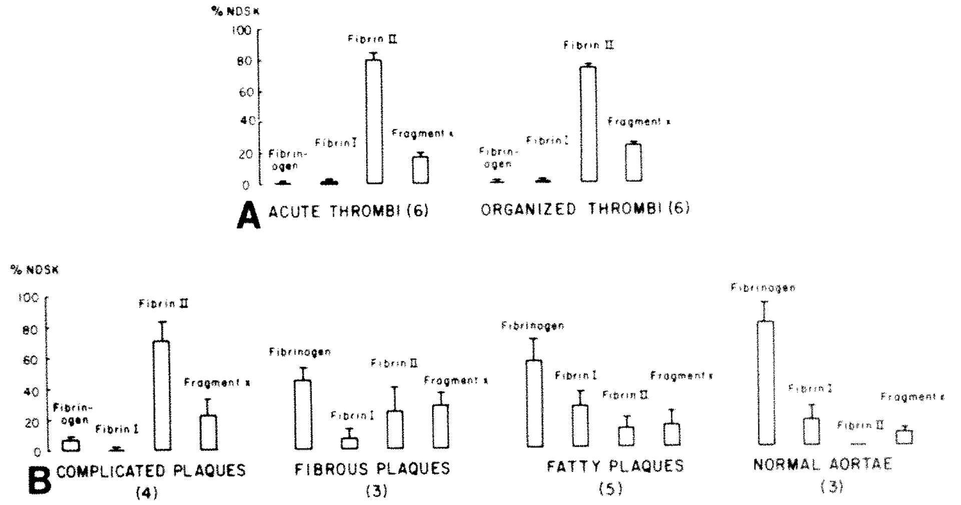

Fig 1. **The relative amounts of fibrinogen, fibrin I, fibrin II, and fragment X [expressed as a percentage of the NH_2-terminal disulfide knot of fibrinogen (NDSK)] for acute and organized thrombi (A), and complicated, fibrous, and fatty plaques and normal aortae (B).**

fibrinogen, fibrin, and fragment X, and CNBr-treated fragment E. Therefore, incomplete recovery of $B\beta 15$-42 in acute and organized thrombi can be assumed to reflect in vivo plasmin cleavage with fragment X formation in both groups. Fragment E formation in vivo would also be detected if the fragment E were noncovalently associated with the fibrin. Whether fragment X was formed by plasmin proteolysis of fibrin I or fibrin II cannot be distinguished in these studies. The comparable recovery of NDSK in the acute and organized thrombi indicates that plasmin cleavage of the NH_2-terminal ends of the fibrin in these thrombi was similar.

Francis and colleagues[7] reported a higher degree of lysis in older thrombi than in more recent ones. They[7] and others[5,6] examined lysis of the carboxy-terminal region of fibrin. Although in vitro studies in this laboratory have shown parallel cleavage of the NH_2-terminal $B\beta$ chains and the COOH-terminal $A\alpha$ chains of fibrinogen,[22] the relationship might be different for fibrin, with or without crosslinking.[41] In addition, the assays used in the present study do not allow distinction between fragment X and fragment E noncovalently associated with the thrombus.

Samples of aortic intima (Table 7) were very different from thrombi in their content of fibrinogen and fibrin-derived fragments. In all groups, the amount of fibrinogen-related protein was <10% of the total protein and was far lower than the 50% to 70% of total protein in thrombi.

The relative amounts of fibrinogen, fibrin I, fibrin II, and fragment X in the complicated plaques were not significantly different from those in the acute and organized thrombi, although the NDSK content was substantially lower. Both FPA and FPB were significantly lower in complicated plaques than in normal aortas or fatty or fibrous plaques, indicating that a significantly higher proportion of the NDSK in the complicated plaques as compared with the other aortic samples was derived from fibrin II. In contrast, the NDSK in normal aortas was derived largely from intact fibrinogen, and that in fatty and fibrous plaques appeared to be intermediate in its derivation from fibrinogen and fibrin I *v* fibrin II. The methods used to obtain the samples and the relatively small numbers of samples with specific diagnoses preclude the drawing of conclusions based on disease states.

The amount of fragment X in the complicated and fibrous plaques was slightly higher than that in the other aortic samples, but the amount of plasmin degradation was not large in any of the histologic types.

Previous studies have reported fibrinogen/fibrin in the aortic intima.[13-15,42] Quantitative comparisons between those studies and the present one are difficult because of methodologic differences in extraction and assay and in calculation and expression of results. In the present study, it has been possible to determine the molecular nature of the fibrinogen present. The total fibrinogen-related antigen is similar in normal aortas and complicated plaques, whereas the proportions of fibrinogen and fibrin II vary inversely with progression of the lesions. These results support the assertion that fibrinogen-related protein was present in the aortic samples in vivo, since in vitro or postmortem acquisition would be expected to show similar proportions of the different antigens. These results also raise the possibility that thrombin proteolysis of fibrinogen occurs within the intima, not only on the vascular surface with subsequent incorporation of fibrin into the intima. Recent studies have shown that fibrinogen can be crosslinked in vitro both by native and activated factor XIII in the presence of calcium ions,[43] and ex vivo studies suggested that the insoluble fibrinogen detected in aortic intima might be crosslinked.[14] Such crosslinked fibrinogen might be a good substrate for thrombin. If proteolysis of fibrinogen does occur within the vessel wall, the question of possible effects of the released fibrinopeptides on vascular endothelial and smooth muscle cells arises. In addition, the effects of cells present in normal aortas and in atherosclerotic aortas on the proteolysis of fibrinogen with the vessel wall must be considered.

Relatively little plasmin proteolysis of the NH_2-terminal end of the intimal fibrin had occurred. A higher degree of plasmin proteolysis of the COOH-terminal $A\alpha$-chains may have occurred; this possibility is being investigated in a companion study. Alternatively, little fibrinolytic activity may occur in the arterial wall, due to low levels of plasminogen activator in both normal and atherosclerotic arteries[44-48] or to high levels of plasminogen activator inhibitor in the arterial wall.[49]

In this study, we demonstrated that thrombi formed in vivo consist of fibrin II as Nossel postulated. The molecular changes in fibrinogen detected in the vessel wall with progression of lesions may result from the triggering of the clotting mechanism in association with the development and/or progression of atherosclerotic lesions. The low degree of plasmin proteolysis of the amino-terminal end of fibrinogen and the fibrin that was detected in both thrombi and plaques suggest either that proteolysis of the carboxy-terminal region occurs preferentially in vivo or that fibrinolysis is limited in the atherosclerotic vessel wall.

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Megakaryocyte Morphogenesis Stimulated In Vitro by Whole and Partially Fractionated Thrombocytopenic Plasma: A Model System for the Study of Platelet Formation

By Robert M. Leven and Marilyn K. Yee

Isolated guinea pig megakaryocytes were cultured in the presence of plasma from normal or thrombocytopenic rabbits. Thrombocytopenic but not normal plasma stimulated formation of long cytoplasmic processes and cytoplasmic fragmentation. Activity was found in the 60% to 80% ammonium sulfate fraction of thrombocytopenic plasma but not in the 0% to 60% fraction. The 60% to 80% fraction of normal plasma contained a small amount of activity. Both colchicine and vincristine inhibited the morphogenesis stimulated by thrombocytopenic plasma. Cytochalasin B and D both mimicked the thrombocytopenic plasma-induced morphological change and affected more megakaryocytes than did the thrombocytopenic plasma. Cytochalasin and thrombocytopenic plasma together had a synergistic effect, causing many megakaryocytes to form processes and break into cytoplasmic fragments 3 to 6 μm in diameter. Immunofluorescence staining with antitubulin antiserum showed that cytoplasmic processes formed in the presence of thrombocytopenic plasma contain microtubules and that fragments released by the megakaryocytes contain microtubule rings. A model for the cytoskeletal basis of platelet formation is proposed.

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THE MATURATION of megakaryocytes culminates in fragmentation of their cytoplasm. The fragments are released into the circulation as newly formed blood platelets. The precise mechanism by which this process occurs is poorly understood. Megakaryocytes have a demarcation membrane system that partitions the cytoplasm of the cells into regions the size of platelets.[1-3] Fusion of these membranes around the platelet areas is believed to lead to the eventual breakup of the cytoplasm into many fragments that cross the endothelium and enter the blood as platelets.[4,5] An alternate mechanism of platelet formation has been proposed. Megakaryocytes in explants of bone marrow will on occasion produce numerous, long cytoplasmic extensions.[6,7] These extensions may have a beaded appearance; consequently, it has been postulated that these cytoplasmic arms break where most attenuated, liberating small fragments, thus forming platelets. This model of platelet formation considers the demarcation membrane system to be a store of membrane rather than a mere demarcation of future platelets.

Not only is the mechanism of platelet formation poorly understood, but neither is it clear whether there is any humoral control of this final stage of megakaryocyte maturation. Based on in vitro studies of megakaryocyte colony formation and in vivo studies on platelet formation, considerable evidence exists for humoral control of megakaryocytopoiesis. Interleukin-3 has been shown to stimulate megakaryocyte colony growth which is potentiated by various conditioned media.[8-10] Yet platelet formation does not normally occur in this type of culture. In vivo studies have shown that certain conditioned media[11] as well as plasma from thrombocytopenic and irradiated animals[12,13] contain an activity that stimulates megakaryocytopoiesis and platelet production when injected into test animals. This activity has been thought to be due to a hormone called thrombopoietin. Thrombopoietin activity has been partially purified from the plasma of thrombocytopenic rabbits,[14] but only one previous investigation has been made of the effect of thrombopoietin-enriched preparations on mature megakaryocytes.[15] This study indicated that DNA synthesis may be stimulated in megakaryocyte cultures when incubated with crude thrombopoietin.

In this study, data are provided to show that a crude thrombopoietin preparation stimulates the formation of long cytoplasmic processes and cytoplasmic fragmentation of isolated guinea pig megakaryocytes. Furthermore, the data provide the basis for a model of the mechanism of platelet formation.

MATERIALS AND METHODS

Megakaryocyte preparation. Megakaryocytes were isolated from Duncan Hartley guinea pig (Camm Laboratories, Wayne, NJ) bone marrow by modification[16] of the method of Levine and Fedorko.[17]

Rabbit plasma preparations. Blood was collected by puncture of the central ear artery from control New Zealand rabbits (R&R Rabbitry, Stanwood, WA) that had undergone no previous manipulations or from rabbits with immune thrombocytopenia. Thrombocytopenia was induced by the intravenous (IV) injection of 2 ml of guinea pig anti-rabbit platelet serum (a gift of Dr S. Ebbe). Prior to injection, a 0.5-ml sample of blood was collected to measure the hematocrit and platelet count. Hematocrits were measured using a Drummond microhematocrit (Drummond Scientific, Broomall, PA). Platelets were counted by the method of Brecher and Cronkite.[18] Four hours after antiserum injection, another 0.5-ml blood sample was drawn. If hematocrits were unchanged and the platelet counts were reduced to <20% of the count prior to antiserum

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injection, the animals were bled by puncture of the central ear artery. Both normal and thrombocytopenic rabbits were bled using $^{1}/_{10}$ vol of 3.8% sodium citrate as an anticoagulant. Blood cells were pelleted by centrifugation at 500 g for 30 minutes. The plasma was centrifuged at 1,500 g for 15 minutes to remove any remaining platelets. The supernatant plasma from the normal (NRP) or thrombocytopenic animals (TRP) was either frozen or further processed as follows. All further steps were performed at 4°C. The plasma was brought to 60% saturation of ammonium sulfate using saturated ammonium sulfate solution with 0.1 mmol/L of etheylene glycol tetracetic acid (EGTA) pH 7.0. The precipitate was collected by centrifugation, and the supernatant was then brought to 80% saturation with ammonium sulfate. The precipitate was again pelleted. The 0% to 60% and 60% to 80% pellets were solubilized in phosphate buffered isotonic saline (PBS) pH 7.0, dialysed against PBS, and concentrated approximately fivefold by dialysis against dry sepharose. The fractionated plasma was then stored in aliquots at −20°C.

Cultures. Isolated megakaryocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 0.1 mg/mL of gentamycin at 37°C in a 5% CO_2–95% air atmosphere. All culture reagents were from GIBCO Laboratories (Grand Island, NY). The cells were generally cultured at 10^5 cells/mL in 35-mm Falcon culture dishes (Falcon Division, Becton Dickinson, Oxnard, CA).

Trypan blue staining. To test viability by exclusion of trypan blue, a sample of the megakaryocytes in culture were mixed with an equal volume of 0.4% trypan blue (GIBCO) in PBS. After 5 minutes, a drop of the cell suspension was placed on a microscope slide and a minimum of 100 cells were counted in each sample. Viable cells were considered to be those that did not stain. In addition, an equal volume of trypan blue solution was added directly to culture dishes to evaluate the staining of megakaryocytes without removing the cells from the culture dish.

^{51}Chromium release. Isolated megakaryocytes in liquid culture at 5×10^5 cells/mL were labeled by incubation with 75 μCi/mL of sodium ^{51}Cr (New England Nuclear, Boston, MA) (specific activity 295.3 mc/mg) for 1 hour at 37°C. After being labeled, the cells were washed three times by centrifugation. The first and second washes were in PBS pH 7.4. The third was in culture medium. The megakaryocytes were resuspended at 250,000 cells/mL in medium and aliquoted at 50,000 cells per well in a 96-well microtiter plate. Test substances were added directly to the wells. For spontaneous release, saline was added at the same volume as for thrombocytopenic and normal plasma fractions. Total release was measured by lysing cells with 3% sodium dodecyl sulfate (SDS). All samples were run in triplicate. The plates were incubated at 37°C for 3 hours, centrifuged at 400 g for 10 minutes; 0.1 mL of the supernatant was removed, and the radioactivity was measured in a TM Analytic Track 1193 γ counter. All measurements were corrected for background counts, and the specific toxicity was determined as 100 × (experimental sample cpm) − (spontaneous release cpm)/(total release cpm) − (spontaneous release cpm).

Immunofluorescence. Antitubulin immunofluorescence was performed by a modification of the method of Osborn and Weber[19] as previously described.[16] Antitubulin antiserum was from Miles Scientific, Naperville IL.

RESULTS

Effect of thrombocytopenic plasma on megakaryocyte morphology. Megakaryocytes were incubated for 18 hours after isolation. Either thrombocytopenic plasma (TRP) or normal rabbit plasma (NRP) was then added at 10% (5 mg/mL of protein) of the culture volume. The 60% to 80% ammonium sulfate fractions of the normal plasma (NRP 60 to 80) or thrombocytopenic plasma (TRP 60 to 80) were added at 1 to 2 mg/mL of final protein concentration; 0% to 60% fractions were added at the same concentration. Preliminary experiments indicated that higher concentrations did not increase the frequency of fragmenting cells; therefore, data were collected in cultures with 1 mg/mL of TRP 60 to 80 or NRP 60 to 80. Cultures were incubated for up to an additional 24 hours under these conditions, but data were collected after 3 hours of incubation since longer incubation times did not increase the number of fragmenting cells. NRP did not cause any change in the normal morphology of the cells; neither did the 0% to 60% fraction of NRP or TRP. NRP 60 to 80 caused a small percentage of the megakaryocytes to undergo fragmentation. TRP and TRP 60 to 80 stimulated fragmentation of a much higher percentage of the megakaryocytes (Table 1). The threefold increase in responsive cells in TRP 60 to 80 at one-fifth the protein concentration indicate an ~15-fold concentration of activity by the ammonium sulfate fractionation. Five hundred to 1,000 cells were counted in each dish to determine the percentage of cells forming processes or fragmenting. Only cells with a nucleus still visible were counted. Cell fragments not associated with a nucleus were not scored.

From 15 minutes to 1 hour after plasma addition, no morphological changes were observed. The first noticeable change in morphology occurred about 1 hour after addition of the TRP or TRP 60 to 80 and appeared to be maximal after 3 to 6 hours. There was considerable cell-to-cell variability in the reaction time. Megakaryocytes began to form processes that initially were 3 to 5 μm in diameter but that with time became much thinner and longer (Fig 1B). Eventually, the central region of the cells also began to partition until the entire cytoplasm was an irregular meshwork (Fig 1C and D). Occasionally, a cell would produce one long process, which could be as long as 500 μm but only 1 to 3 μm in diameter (Fig 1E). The processes often appeared beaded

Table 1. Effect of TRP and TRP 60–80 on Megakaryocyte Fragmentation

Culture Condition	Percentage of Megakaryocytes Forming Processes
Control (no additions)	0
NRP (10%)	0.1 ± 0.14*
TRP (10%)	5.4 ± 0.58
NRP 60–80 (1.0 mg/mL)	1.0 ± 0.82
TRP 60–80 (1.0 mg/mL)	18.4 ± 1.58
TRP 60–80 + 1 mg/mL of colchicine	5.0 ± 0.69
TRP 60–80 + 10 μg/mL of vincristine	3.7 ± 0.80

TRP, thrombocytopenic rabbit plasma; NRP, normal rabbit plasma.

Data are the averages from three experiments in which a minimum of 500 cells was counted for each condition. Each experiment was from a different megakaryocyte preparation. Colchicine and vincristine were added 1 hour prior to addition of TRP 60–80. Counts were made 3 hours after NRP, TRP, or TRP 60–80 addition. TRP was significantly greater than NRP ($P < .001$). TRP 60–80 was significantly greater than NRP 60–80 ($P < .005$). Inhibition was significant by both colchicine ($P < .01$) and vincristine ($P < .005$) as compared with TRP 60–80 alone.

*SEM.

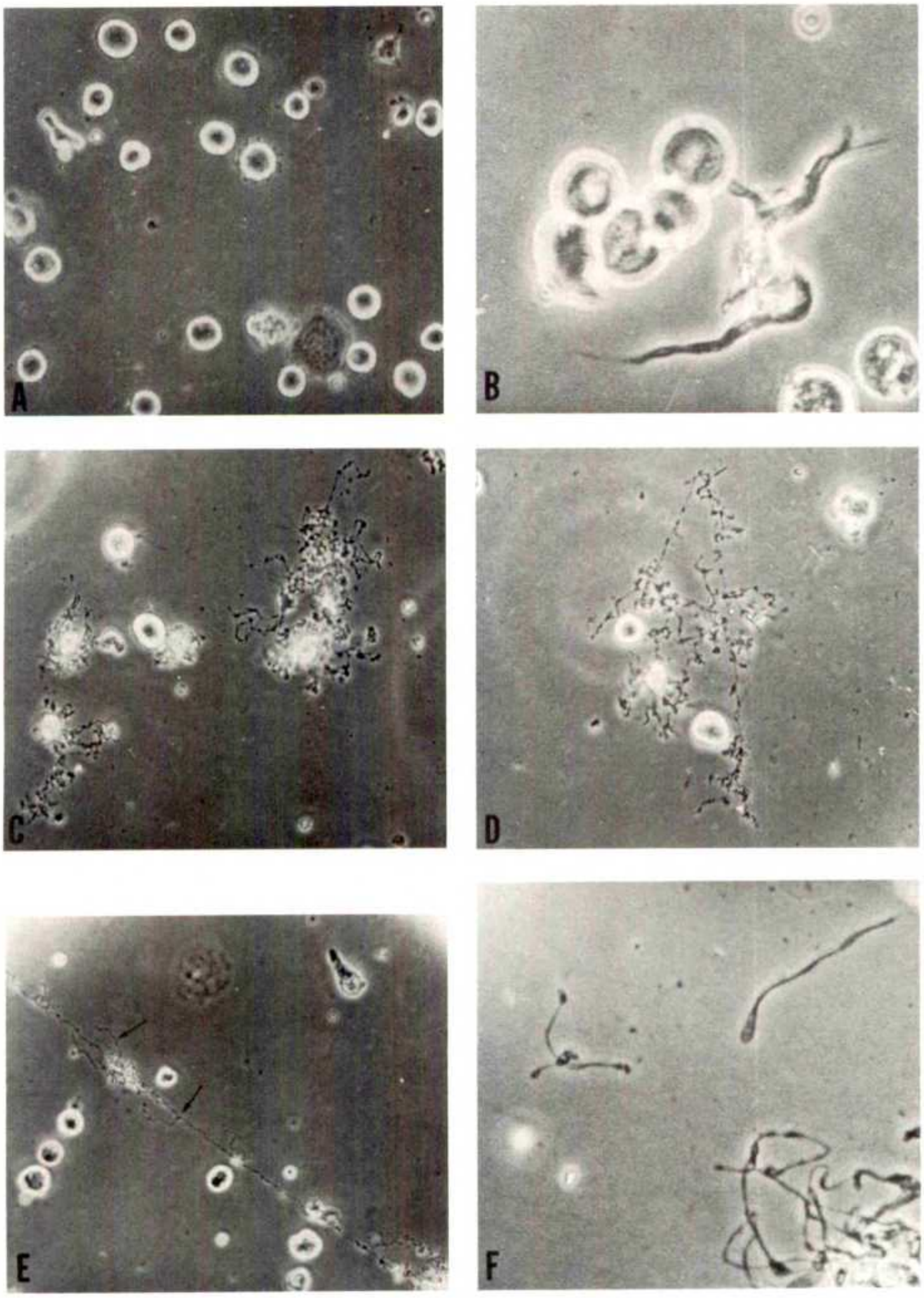

Fig 1. Response of megakaryocytes to the 60% to 80% ammonium sulfate fraction of thrombocytopenic rabbit plasma (TRP). Phase micrographs of isolated guinea pig megakaryocytes in liquid culture with 1.0 mg/mL of TRP 60 to 80. (A) Control megakaryocytes, no TRP 60 to 80 added to the culture. Cells maintained their spherical shape in control cultures until the cultures terminated due to senescence after 3 to 4 days. (Original magnification ×98.5.) (B) Megakaryocyte beginning to respond 1 hour after thrombocytopenic serum addition to the culture. Large cytoplasmic processes are beginning to form. (Original magnification ×395.) (C) through F) After 3-hour incubation in TRP 60 to 80. (C) Several megakaryocytes at a later stage of fragmentation. The entire cytoplasm is beginning to partition in these cells. (Original magnification ×60.) (D) Megakaryocyte prior to fragmentation. The cytoplasm is fully partitioned, and beading is evident along the cytoplasmic processes. (Original magnification ×160.) (E) Example of a megakaryocyte that has produced one very long cytoplasmic process. Several short branches are visible along the major process (arrows). (Original magnification ×175.) (F) Cytoplasmic fragments that have just been released by a megakaryocyte that is beginning to fragment. (Original magnification ×255.)

due to irregularly spaced thickenings clearly seen in the longer processes. Some cells eventually broke into fragments. On occasion, large fragments broke into smaller fragments (Fig 1F). Because the cells were in liquid culture, the smaller fragments were quickly carried away from the main cell mass. After 24 hours, few fragmenting cells were observed, possibly due to break up the fragmenting cells. Anti-rabbit platelet antiserum was added directly to cultures at a 1:75 dilution. This is comparable to the antibody dilution in a 3-kg rabbit with a 150-ml blood volume.[20] Cells were incubated with antiserum for up to 6 hours, and no change in cell morphology was observed.

Cell viability. Cell viability was measured in two ways; trypan blue staining and ^{51}Cr release. Megakaryocytes were incubated with TRP 60 to 80 or NRP 60 to 80 or with saline as a control. Table 2 shows that neither of these plasma fractions altered cell viability when measured by either trypan blue exclusion or ^{51}Cr release.

Effect of colchicine, vincristine, and cytochalasins. Megakaryocytes were incubated with either colchicine (1 mg/mL) or vincristine (10 μg/mL) for 1 hour prior to the addition of TRP 60 to 80. After additional incubation with TRP 60 to 80 for 3 hours, the percentage of fragmenting cells was determined. Neither colchicine nor vincristine alone altered megakaryocyte morphology, nor did 1.0% ethanol, the solvent for colchicine. When cultures were preincubated with these drugs prior to the addition of TRP 60 to 80, however, the number of fragmenting cells was sharply

Table 2. Effect of NRP 60–80, TRP 60–80 Colchicine and Vincristine on Megakaryocyte Viability

	Percentage Viable	
Trypan Blue Exclusion		
Control (no addition)	86.5 ± 1.36*	
NRP 60–80 (1.0 mg/mL)	83 ± 0.94	
TRP 60–80 (1.0 mg/mL)	85 ± 0.95	
Colchicine (1.0 mg/mL)	80 ± 0.77	
Vincristine (10 μg/mL)	89 ± 1.63	
	Specific Toxicity	
^{51}Chromium release	Exp 1	Exp 2
NRP 60–80 (1.0 mg/mL)	0	0
TRP 60–80 (1.0 mg/mL)	0	0.4

Abbreviations as in Table 1.

Data from the trypan blue exclusion experiments are average results of three experiments. Measurements are made in triplicate for each condition in each experiment. Viability in NRP 60–80 and TRP 60–80 with trypan blue was determined after 3-hour incubation. Viability in colchicine and vincristine was determined 3 hours after drug addition. None of the groups show any significant difference from the control ($P > .05$). Specific toxicity by Cr release was determined as described in the Materials and Methods section. Data show the results of two experiments in which measurements for each condition were made in triplicate. Cr release was measured after 3-hour incubation in TRP 60–80 or NRP 60–80.

*SEM.

decreased (Table 1). If the drugs were added after the 3-hour incubation with TRP 60 to 80, limited retraction of processes occurred. Neither drug affected cell viability, as determined by trypan blue exclusion (Table 2).

Cytochalasins had a very different effect on megakaryocyte morphology. Both cytochalasin B (10 μg/mL) and cytochalasin D(2 μg/mL) caused cytoplasmic process formation and partitioning of the megakaryocyte cytoplasm similar to that caused by TRP and TRP 60 to 80, (Fig 2). The morphogenesis with cytochalasins also occurred in 1 to 3 hours. There were two notable differences in the response to the cytochalasins. First, many more cells formed processes after incubation in the cytochalasins than in TRP or TRP 60 to 80. The percentage of cells responding was difficult to count accurately since many responding cells overlapped; thus, distinguishing individual cells was not always possible. Second, the cytoplasmic processes formed during exposure to cytochalasins tended to have a very smooth contour rather than the irregular beading that occurred in the presence of TRP or TRP 60 to 80. When megakaryocytes were incubated with either cytochalasin in the presence of TRP or TRP 60 to 80, there was an increase in the number of the megakaryocytes that formed processes with distinct beading

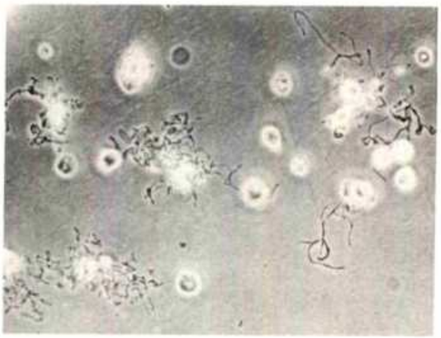

Fig 2. Isolated megakaryocytes in culture exposed to 2 μg/mL of cytochalasin D for 3 hours. Many megakaryocytes have formed cytoplasmic processes. (Original magnification ×250.)

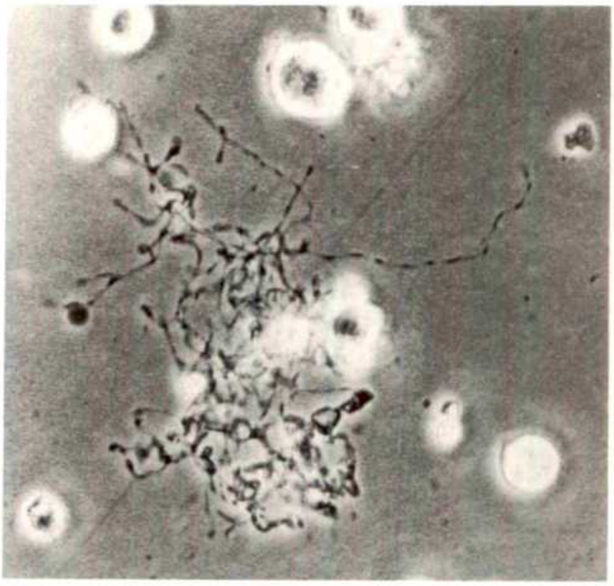

Fig 3. Megakaryocytes incubated with 2 μg/mL of cytochalasin D and 1.0 mg/mL of thrombocytopenic rabbit plasma (TRP) 60 to 80 for 3 hours. Many long processes have formed, and extensive partitioning of the entire cytoplasm has occurred. Beading is clearly apparent along the processes. (Original magnification ×275.)

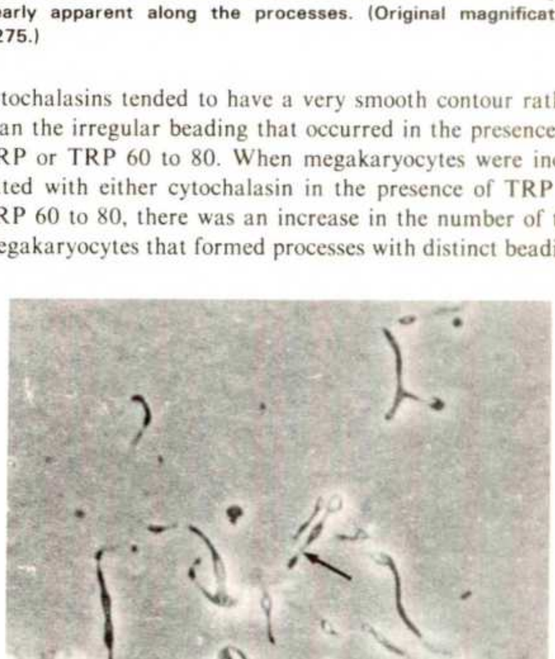

Fig 4. Megakaryocytes incubated for 3 hours with 1.3 mg/mL of thrombocytopenic rabbit plasma (TRP) 60 to 80 and 2 μg/mL of cytochalasin D. Several large proplatelet fragments have formed. Beading is evident on some fragments. (Original magnification ×250.) Many cytoplasmic fragments are visible. The diameters of fragments range from ~3 to 6 μm, and many are still attached by thin cytoplasmic threads (arrows).

(Fig 3). The cells appeared similar to those in TRP alone except for the increase in the number of responsive cells. Megakaryocytes commonly broke into large and small fragments under these conditions (Fig 4).

Tubulin immunofluorescence. Fragmenting megakaryocytes were observed by indirect immunofluorescence using antitubulin antiserum. The cytoplasmic processes were found to contain many microtubules extending along the long axis of the processes. The partitioning cytoplasm in the central area of the cell also contained microtubules. Microtubule rings were seen both in processes and near the cell body. Cytoplasmic fragments containing microtubule rings were also observed (Fig 5).

DISCUSSION

These results show that plasma obtained from rabbits with immune thrombocytopenia contains a factor that stimulates cytoplasmic fragmentation of isolated megakaryocytes. Thrombocytopenia has been shown to increase megakaryocyte maturation rate, number, size, and ploidy.[21-24] These changes occur over several days and are believed to depend on alteration of immature megakaryocytes and precursor cells. It has, however, been shown that a burst of large platelets is released within 18 hours of induced thrombocytopenia.[22] This more rapid response indicates that mature megakaryocytes may be directly affected in a thrombocytopenic state. The type of thrombocytopenic plasma preparation that we used stimulates platelet production in vivo,[12] but it is not clear whether mature megakaryocytes can be directly affected by thrombocytopenic plasma.

The megakaryocytes we isolated from bone marrow are recognizable megakaryocytes and not megakaryocytic precursors. Therefore, our observations show that megakaryocytes in the final stages of maturation are capable of responding to a factor that is increased in thrombocytopenic plasma within 4 hours of antiplatelet serum injection. Only one previous study investigated the effect of a thrombocytopenic plasma preparation on isolated megakaryocytes.[15] Stimulation of DNA synthesis was reported to occur during incubation in TRP 60 to 80; however, no observations were made on cell morphology. In our studies, up to 18% of the megakaryocytes fragment with TRP or TRP 60 to 80; therefore, it is possible that only a particular subpopulation of megakaryocytes responds to TRP. It would be interesting to know if megakaryocytes of a particular maturation stage or ploidy are the most responsive.

No fragmentation occurred when the cells were incubated with normal rabbit plasma, although a small amount did

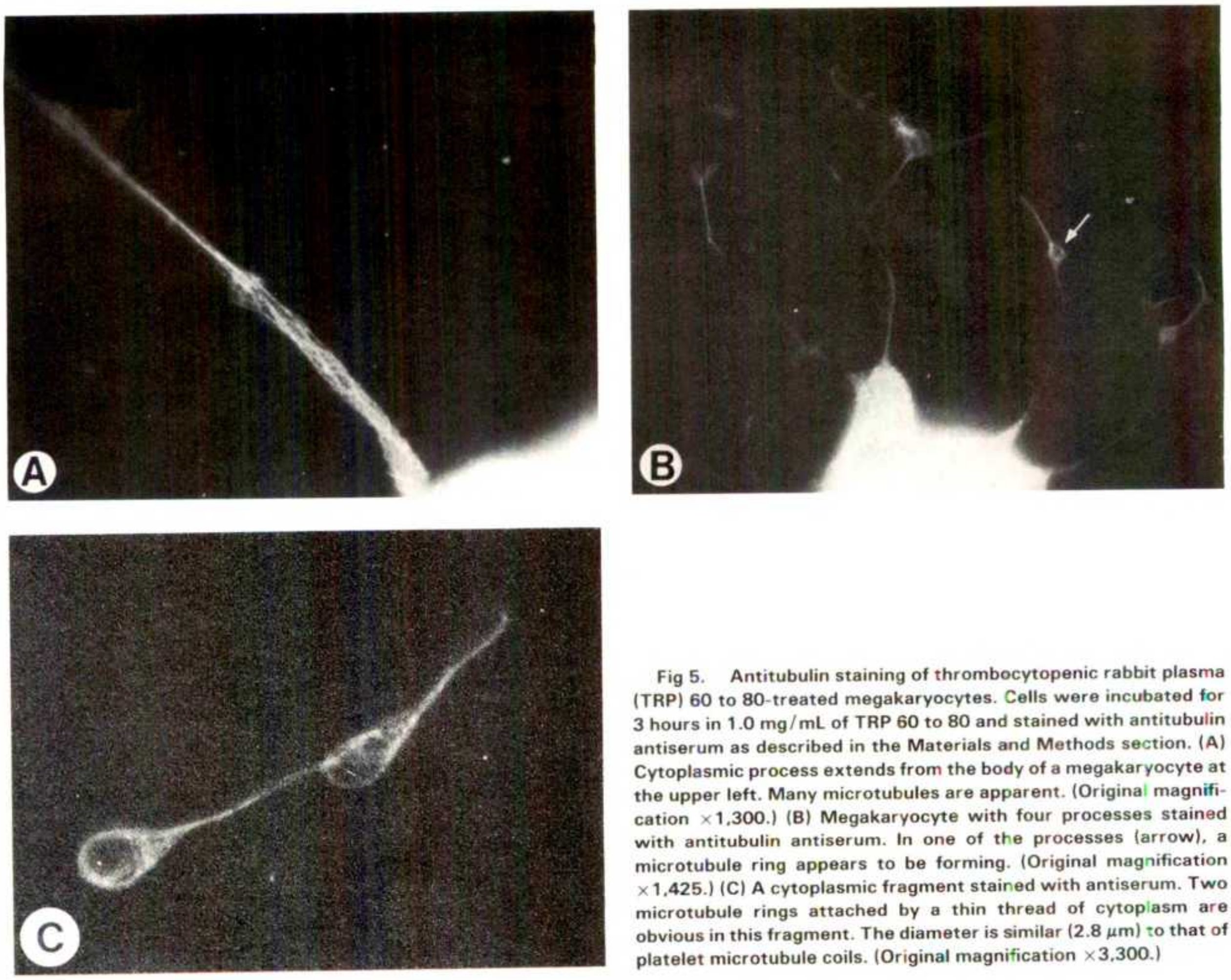

Fig 5. Antitubulin staining of thrombocytopenic rabbit plasma (TRP) 60 to 80-treated megakaryocytes. Cells were incubated for 3 hours in 1.0 mg/mL of TRP 60 to 80 and stained with antitubulin antiserum as described in the Materials and Methods section. (A) Cytoplasmic process extends from the body of a megakaryocyte at the upper left. Many microtubules are apparent. (Original magnification ×1,300.) (B) Megakaryocyte with four processes stained with antitubulin antiserum. In one of the processes (arrow), a microtubule ring appears to be forming. (Original magnification ×1,425.) (C) A cytoplasmic fragment stained with antiserum. Two microtubule rings attached by a thin thread of cytoplasm are obvious in this fragment. The diameter is similar (2.8 μm) to that of platelet microtubule coils. (Original magnification ×3,300.)

occur with NRP 60 to 80. It is possible that concentration of a reduced level of thrombopoietin which circulates in normal plasma was responsible for the small number of fragmenting cells. This is consistent with the observation that thrombopoietin activity can be detected in normal plasma when injected into platelet-hypertransfused test animals.[25,26] The fragmentation that occurs does not appear to be due to cellular toxicity. Using two independent methods to measure cell viability, we were unable to detect any decrease of viability in either NRP 60 to 80 or TRP 60 to 80.

The morphological change that occurs in megakaryocytes incubated with TRP or TRP 60 to 80 appears similar to the spontaneous morphogenesis of megakaryocytes observed by Thiery and Bessis[6] and by Radley and Haller.[7] Unlike these investigators, we observed no spontaneous process formation. In the absence of TRP and TRP 60 to 80, no morphological changes occurred in our cultures. The previous studies used teased marrow explants rather than isolated megakaryocytes. It may therefore be the presence of other marrow cells or factors in the marrow that allowed this spontaneous activity to occur.

On the basis of our findings and previous findings, we believe that megakaryocyte process formation is a microtubule-dependent phenomenon. Radley and Haller[7] observed that spontaneous cytoplasmic process formation was reversed by vincristine, and in our studies both colchicine and vincristine inhibited the TRP-induced morphogenesis. In addition, tubulin immunofluorescence showed that the cytoplasmic processes are microtubule-containing structures. Ultrastructural studies have shown that megakaryocytes in the marrow can form processes[27,28] containing aligned microtubules. Therefore, the formation of microtubule-based cytoplasmic processes is not likely to be an artifact of culture conditions but may be an important step in platelet formation. The presence of microtubule rings further supports the idea that the in vitro morphogenesis we observed is similar to platelet formation in vivo, particularly since the rings are seen not only in the cytoplasmic processes but also in some released fragments that are the size of platelets. We do not know if the fragments released are functional platelets, but the presence of microtubule rings implies that structural organization of the platelet cytoskeleton may occur at the time of, or possibly prior to, cytoplasmic fragmentation.

The cytoplasmic process formation and partitioning that occurred with cytochalasin was similar to that which occurred with TRP and TRP 60 to 80. The smooth surface of processes that form with cytochalasin indicate that TRP-induced and cytochalasin-induced morphogenesis may not be identical. Moreover, cytochalasins caused far more cells to begin fragmentation than did TRP or TRP 60 to 80. The active factor in TRP may stimulate megakaryocytes by a surface receptor that is not expressed on all cells. Because the cytochalasins diffuse into the cells and do not depend on a surface receptor, one would expect more cells to be affected.

From the data presented, we propose a model for the cytoskeletal basis of platelet formation. The factor stimulating fragmentation in TRP binds to a receptor on the surface of certain megakaryocytes, perhaps the most mature. Binding then triggers a change in the organization of actin in the cell. Actin filaments or filament networks, which maintain the cytoplasm intact, disassemble, causing the cytoplasm to change from a gel-like to sol-like state as postulated to occur in ameboid cells.[29] The disassembly of microfilament structures may be regulated by the actin regulatory proteins that have been identified in platelets[30-33] and probably exist in megakaryocytes as well. The processes that subsequently form have a core of microtubules and may elongate by polymerization of tubulin. We believe the long fragments of cytoplasm that form are comparable to the proplatelet processes observed by Behnke[23] and by Becker and De Bruyn.[34] What controls the fragmentation of proplatelets into platelets is still unexplained. A microfilament-based contractile mechanism is not likely to be involved since fragmentation occurs in the presence of cytochalasins. It is possible that other structural components determine the interval at which cytoplasmic processes fragment. The microtubule rings may play a role in controlling the intervals at which proplatelets break into platelets. Our model is similar to that of Becker and De Bruyn's interpretation of platelet formation,[34] although based on our results with megakaryocytes incubated with cytochalasins we do not believe actomyosin contraction is involved in forming processes as Becker and DeBruyn have proposed. Although speculative, our model of platelet formation is consistent with available data, and we believe it provides a useful basis for further study of the mechanism of platelet formation.

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Stimulation by cAMP of Erythropoietin Secretion by an Established Human Renal Carcinoma Cell Line

By J.B. Sherwood, E.R. Burns, and D. Shouval

We used our recently reported stable, transformed human renal carcinoma cell line as a model system to study the role of 3′, 5′-adenosine monophosphate (cAMP) in erythropoietin secretion. The erythropoietin produced by these cells is both biologically active and immunologically cross-reactive with purified native human hormone in our radioimmunoassay. Erythropoietin release by these renal carcinoma cells appears to be stimulated by cAMP as well as by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX). The response to cAMP involves a rapid and enhanced release of hormone, which occurred within 30 minutes of exposure of the cells to the effector and continued for at least 4 hours. Intracellular erythropoietin was higher in the control cultures than in the cells treated with cAMP, suggesting that cAMP stimulates the release of a storage pool of hormone. The ability of cAMP and MIX to elicit the release of erythropoietin suggests that a cAMP-mediated mechanism is involved in the release of this hormone.

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WE RECENTLY described[1] the development of a stable, transformed human renal clear cell carcinoma cell line that produces erythropoietin in vitro and that has maintained this function for several years and for >150 passages in culture. The erythropoietin produced by these cells appears to be biologically active in both the in vitro mouse CFU-E assay and in nude mice carrying the tumor. The tumor-produced hormone is detectable in our radioimmunoassay, and its ability to displace pure native human urinary hormone from the antierythropoietin antibody indicates that it shares structural similarities with the native protein.[1]

Considerable evidence supports a correlation between erythropoietin production in vivo and such parameters involved in oxygen supply as hemoglobin concentration, RBC mass, and ambient pO_2.[2] Little information exists, however, on regulation of erythropoietin synthesis and secretion at the cellular level. Our stable, established renal carcinoma cell line provides an excellent model system for studies of erythropoietin production at the cellular level. It also allows a comparison of regulation of hormone release by carcinoma *v* normal cells.

Cyclic 3′, 5′-adenosine monophosphate (cAMP) is known to regulate the release of many polypeptide hormones.[3] This effect has been observed in vitro for a number of hormones such as parathyroid hormone,[4-6] thyroid hormone,[7] and growth hormone.[8-10] A "first messenger" has been shown to act at the cell membrane to stimulate the membrane-bound adenyl cyclase, resulting in conversion of ATP to intracellular cAMP, which then acts to mediate hormone release.[3] In the case of erythropoietin, decreased oxygen levels may serve as a first messenger. We are therefore using our renal carcinoma cell line to examine the hypothesis that cAMP mediates an extracellular stimulus to erythropoietin production.

MATERIALS AND METHODS

Cells. The human renal carcinoma cell line designated as the RC-1 line was used for all studies. This line was derived from a renal cell carcinoma obtained in 1981 from a patient with erythrocytosis. These cells grow as a homogeneous monolayer, with neoplastic characteristics as evidenced by loss of contact inhibition and by the ability to be cloned and grown in soft agar.[1,11] The characteristics of this cell line have been fully described.[1] The current studies were performed on cells from several different passages (passages 37 through 105).

Cell culture methods. Tumor cells were routinely maintained in Corning tissue culture flasks (Corning Glass, Corning, NY) in complete medium, consisting of Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F12 (Ham) 1:1 by volume supplemented with 10% fetal bovine serum (FBS), 2 mmol/L of L-glutamine, 0.10 mmol/L nonessential amino acids, 100 U of penicillin, and 100 µg of streptomycin. All media components were obtained from Grand Island Biological (Grand Island, NY).

Radioimmunoassay of erythropoietin. Erythropoietin levels in the culture supernatant fluids and the cell extracts were determined in the radioimmunoassay described by Sherwood and Goldwasser.[12] The pure erythropoietin used as the radiolabeled tracer was described by Miyake and co-workers[13] and was generously supplied by Dr Eugene Goldwasser (University of Chicago) and by the Blood Diseases Branch, Division of Blood Diseases and Resources of the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD. The erythropoietin was radiolabeled with ^{125}I, using the water-insoluble oxidizing agent 1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycoluril (Iodogen, Pierce Chemical, Rockford, IL),[14] to a specific activity of 16 to 21 μCi $^{125}I/\mu g$ of erythropoietin. The antibodies to erythropoietin were raised in New Zealand White rabbits in response to a preparation of crude human urinary erythropoietin. Human urinary erythropoietin (Toyobo, Osaka, Japan) was used as the standard in the assay. The methods for incubation of samples and antibody and for separation of antibody-bound and free erythropoietin by the second antibody technique were described previously.[12] The ratio of bound to total ^{125}I-erythropoietin was determined for the standard curve and for the culture and serum samples. All erythropoietin standards and unknown samples were assayed in triplicate. Medium with and without the experimental additives incubated without cells served as assay controls. Values are expressed as mean ±SEM, representing the replicate cultures.

Preparation of samples for detection of erythropoietin. Supernatant fluids obtained from culture dishes were centrifuged for 10 minutes at 1,000 rpm at 4°C in an IEC Centra 7-R tabletop

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refrigerated centrifuge (International Equipment, Needham Heights, MA). The culture fluid was then stored at −20°C to −70°C until assay.

Cells were harvested from the cultures with 0.1% trypsin-0.1 mol/L of EDTA, washed with Earle's balanced salt solution (BSS), and extracted in 0.1 mol/L of phosphate buffer by homogenization with a Polytron (Brinkmann Instruments, Westbury, NY) as described by Sherwood and Goldwasser.[15] The cell extracts were stored at −20°C to −70°C until assayed.

Evaluation of effect of cAMP on erythropoietin production. For studies of erythropoietin production, cells were removed from the surface of the flasks by incubation with 0.1% trypsin-0.1 mol/L of EDTA, followed by resuspension in complete medium. The number of viable cells was determined in a hemocytometer, using exclusion of trypan blue dye as an indicator of viability. Each 60-mm Falcon tissue culture dish (Becton Dickinson, Cockeysville, MD) was seeded with 3×10^5 viable cells in 5 mL of complete medium and incubated at 37°C in 5% CO_2-95% air until confluence (1 to 2×10^6 cells per plate) was reached. The cultures were then incubated at 37°C in 5% CO_2-95% O_2 in a humidified tissue culture incubator (Forma Scientific, Marietta, OH) in 2 mL of "complete medium" containing 5% fetal bovine serum (FBS), and 8-bromo cAMP or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX) as indicated. Control cells from the same passage were incubated in the same medium (5% FBS), without cAMP or MIX, at the same time. Three replicate cultures were made for each effector concentration and time point. At the indicated times, cells and supernatant media were harvested, the viable cell count was obtained, and the erythropoietin was extracted from the cells as described. Erythropoietin levels in the media and cell extracts were determined by radioimmunoassay.

RESULTS

Effect of cAMP on intracellular and extracellular erythropoietin. Concentrations of 8-bromo cAMP $<10^{-5}$mol/L were ineffective in stimulating erythropoietin production. Therefore, in these studies, cells were incubated in experimental medium with or without 10^{-4}mol/L of 8-bromo cAMP for periods of 1 to 4 hours. As shown in Table 1, erythropoietin levels in the supernatant media from cAMP-treated cells were significantly higher ($P < .02$) than in the media from control cultures from the same passage incubated (at the same time) without cAMP. Conversely, erythropoietin levels in the cell pellets (intracellular erythropoietin) were significantly ($P < .02$) lower in the cAMP-treated cultures than in the controls. Control medium with and without 10^{-4}mol/L of 8-bromo-cAMP, incubated without cells, contained nondetectable amounts of erythropoietin (<10 mU/mL). The cAMP appeared to have no effect on cell morphology, growth, or viability.

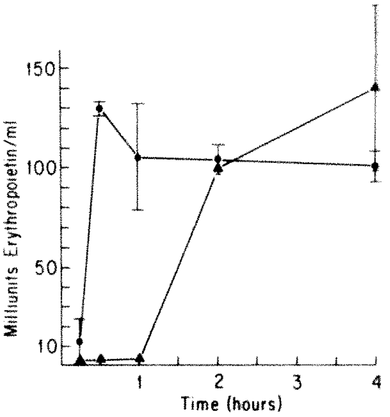

Fig 1. Effect of cAMP on the time course of erythropoietin secretion by the human renal carcinoma cell line: Cells from passage 105 were grown to confluence (~6 × 10^6 cells/60-mm plate) and incubated in standard culture medium with (●—●) or without (▲—▲) 10^{-4}mol/L of 8-bromo cAMP. At the times shown, the supernatant medium was harvested from three plates. Erythropoietin levels were measured in the radioimmunoassay. Each data point represents the mean for the replicate cultures, each measured in triplicate (SD generally ±10%).

Effect of 8-bromo-cAMP on time course of erythropoietin release. Cells from passage 105 were incubated in experimental medium with or without 10^{-4}mol/L of 8-bromo-cAMP. As shown in Fig 1, a slight secretory response to 10^{-4}mol/L of 8-bromo-cAMP was observed within 15 minutes (13 ± 10 mU/mL of medium) and reached a maximum at 30 minutes (130 ± 3 mU/mL). Erythropoietin levels in

Table 1. Effect of 8-Bromo-cAMP on Intracellular and Extracellular Erythropoietin

Experiment No.	Cell Passage No.	Effector	Sample Assayed	Incubation (hours)	Erythropoietin (mU per 10^6 Cells)
1	37	cAMP	Supernatant	4	110 ± 1
			Cells		118 ± 39
		Control	Supernatant	4	ND*
			Cells		310 ± 38
2	86	cAMP (10^{-4} mol/L)	Supernatant	1	605 ± 123
			Cells		ND*
		Control	Supernatant	1	111 ± 17
			Cells		136 ± 40
3	87	cAMP (10^{-4} mol/L)	Supernatant	2	1143 ± 88
			Cells		37 ± 5
		Control	Supernatant	2	71 ± 18
			Cells		540 ± 40

Cells were incubated at confluence with 10^{-4} mol/L of 8-bromo cAMP in 2 mL of F12:Dulbecco's modified Eagle's medium. Erythropoietin values were determined in the radioimmunoassay. Each value represents the mean ± SEM for three plates, each measured in triplicate. All values are significantly different from values for control cells.

*ND, not detectable, <10 mU/mL. Control medium without cells had no detectable erythropoietin.

the medium remained constant for at least 4 hours (1 hour, 108 ± 27 mU/mL; 2 hours, 107 ± 7 mU/mL; 4 hours, 98 ± 8 mU/mL). The values for these time periods did not differ significantly ($P = .09$) from the 30-minute value. In contrast, erythropoietin was not detected in the supernatant medium of control cultures of the passage 105 cells until 2 hours of incubation (103 ± 3 mU/mL for 2 hours, 142 ± 42 mU/mL for 4 hours). The 4-hour value for the control cultures did not differ significantly ($P = .27$) from that of the cAMP-treated cultures from the same passage, suggesting some constitutive release by cells of this passage. No erythropoietin was detectable in the control media with and without cAMP, incubated without cells.

Effect of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine on erythropoietin release. Cultures of passage-37 cells were incubated for 4 hours in experimental medium with 10^{-4}mol/L of MIX. After the 4-hour incubation period, the supernatant fluids were harvested; erythropoietin levels in the samples and in control medium containing the MIX but not incubated with cells were measured in the radioimmunoassay. Addition of either MIX or cAMP to the cultures was associated with erythropoietin levels in the supernatant medium that were significantly greater than in the control cultures: cultures with 10^{-4}mol/L of MIX, 48 ± 4 mU erythropoietin/10^6 cells; cultures with 10^{-4}mol/L of 8-bromo cAMP, 110 ± 1 mU/10^6 cells; control cultures, no detectable erythropoietin; and no detectable erythropoietin in the control medium incubated without cells.

Effect of long-term incubation on erythropoietin production by passage-37 cells. Cells from the passage 37 were incubated in experimental medium with and without 10^{-4}mol/L of 8-bromo cAMP, and the supernatant fluids were harvested after 4 hours and 24 hours of incubation. After 4 hours of incubation, erythropoietin levels in the supernatant media from cAMP-treated cells (110 ± 1 mU/10^6 cells) were significantly higher ($P < .02$) than in the medium from control cultures from passage 37 incubated without cAMP (nondetectable erythropoietin levels), whereas intracellular erythropoietin was higher in the controls (310 ± 38 *v* 118 ± 39 mU/10^6 cells). Control passage-37 cells did not appear to show constitutive erythropoietin release until 24 hours of culture; after 24 hours of incubation, erythropoietin levels in the medium were 38 ± 14 mU/10^6 cells in cAMP-treated cultures and 34 ± 10 mU/10^6 cells in controls (cAMP *v* control, P = NS). The cell content was higher in the cAMP-treated cultures (474 ± 84 mU/10^6 cells), however, than in the controls (118 ± 10 mU/10^6 cells).

DISCUSSION

This report describes the effect of 8-bromo-cAMP on the production of erythropoietin in vitro by our stable, transformed human renal carcinoma cell line[1] and provides evidence that cAMP is an intermediate in the regulation of erythropoietin production.

We described this cell line in a recent publication.[1] The cells have been maintained in culture since 1981 and for >150 passages and retain an abnormal human karyotype, ultrastructural features characteristic of human renal clear cell carcinoma, and the growth characteristics of transformed neoplastic cells.[1] The erythropoietic factor produced by this cell line appears to be biologically active in vitro, in the mouse CFU-E assay,[1] and in vivo, as demonstrated by the development of significant erythrocytosis in athymic Balb/c mice transplanted with cells from this line.[16] In our radioimmunoassay, displacement curves produced by increasing amounts of culture supernatant fluids were parallel to those produced by pure native human urinary erythropoietin, suggesting that the native human hormone and the tumor factor are similar.[1]

Little information exists on regulation of erythropoietin synthesis and secretion at the cellular level, in part due to the difficulty of establishing a suitable in vitro model system. Previously reported in vitro systems include cultures of normal kidney cells such as goat renal glomeruli[17] and rat mesangial cells,[18] as well as transformed cells such as the human renal carcinomas reported by Hagiwara and colleagues,[19,20] Sherwood and Goldwasser,[21] Sherwood and Shouval,[1,22] and Sytkowski and coworkers,[23] a human testicular carcinoma,[24] and mouse erythroleukemia cells.[25] Our studies represent the first reported use of a long-term stable human cell line to study regulation of erythropoietin production at the cellular level.

The membrane enzyme adenylate cyclase and its product cAMP are intermediates in the regulation of secretion of some polypeptide hormones.[3] The secretory effects of cAMP are mediated through activation of a cAMP-dependent protein kinase, leading to exocytosis of secretory granules.[26]

Erythropoietin production by our renal carcinoma cell line was stimulated by 8-bromo-cAMP and MIX. MIX, a cyclic nucleotide phosphodiesterase inhibitor, prevents the degradation of intracellular cAMP, thus allowing its accumulation.[5] The 8-bromo-cAMP used in these studies is a derivative of cAMP with an 8 substitution of the purine ring. Although these derivatives are able to inhibit cyclic nucleotide phosphodiesterase and to activate cAMP-dependent protein kinase, their primary mechanism of action at the cellular level is considered to occur by protein kinase activation.[27]

In these studies, cells were incubated with and without cAMP or with and without MIX, and erythropoietin production by cells of the same passage was measured by radioimmunoassay. Comparisons were made between cells of the same passage, not between cells of different passages. In all experiments and cell passages, erythropoietin levels in medium from cultures incubated with either 8-bromo-cAMP or with MIX were significantly higher than in medium from the control cultures of the same passage. In the short-term cAMP-treated cultures, the increased erythropoietin in the medium was associated with a concomitant decrease in intracellular hormone, in contrast to the control cultures—which had high intracellular and low media levels of erythropoietin.

cAMP produced a rapid and enhanced release of erythropoietin with 15 minutes of exposure of the cells to the nucleotide. Erythropoietin release continued to increase for ~30 minutes, after which the levels in the medium remained relatively constant (erythropoietin levels at the other times did not differ significantly from the 30-minute value). Erythropoietin release after 2 hours by the control cells of this passage suggests some constitutive production.

This pattern of erythropoietin release in response to elevated intracellular cAMP is similar to the response that has been observed for other hormones.[3,4-10,28] Addition of dibutyryl (db) cAMP at concentrations of $\geq 10^{-4}$mol/L to parathyroid gland explants resulted in significant stimulation of parathyroid hormone release 1 hour after incubation.[4,6] Growth hormone release from bovine pituitary gland anterior lobe explants[10] and cultures of rat adenohypophyseal somatotrophs[9] was stimulated within 30 minutes in response to elevated extracellular and intracellular cAMP and remained relatively constant during the 2-hour incubation period. cAMP also stimulates release of thyroid hormone,[7] thyroid-stimulating hormone,[8] insulin,[28] and calcitonin[28] in similar fashion.

cAMP stimulation may also lead to increased synthesis of some proteins.[3] In each experiment the total amount of erythropoietin in each culture (cellular content plus released hormone) was higher in the cAMP-treated culture ($P < .05$). This effect occurred in the cultures of passages 86 and 87 after 1 and 2 hours of incubation. Passage 37 cultures had similar total erythropoietin levels after a 4-hour incubation period. After 24 hours, however, the cAMP-treated cultures had a significantly higher intracellular erythropoietin content, but cAMP and control cultures had similar levels in the medium. Although cAMP effects are short-range phenomena in endocrine systems, cAMP may have some long-term effects on erythropoietin production[29,30] and on erythropoiesis.[31]

The increase in intracellular erythropoietin in the cAMP-treated cultures may be due to direct stimulation of synthesis by cAMP or may involve a feedback stimulus caused by depletion of an intracellular pool by the cAMP-induced hormone release. There may also be two intracellular pools of erythropoietin responsive to different physiological stimuli, one pool responsive to agents that elevate cellular cAMP and another pool responsive to other factors, as suggested also for parathyroid hormone.[32,33] As support for this, we showed that this cell line produces erythropoietin under control conditions (Table 1 and Fig 1), an observation that was important to us in developing this erythropoietin-producing line[1] in amounts and at time intervals that may differ for each cell passage. Some erythropoietin may also be released in a constitutive fashion, as has been reported for other culture systems.[1,19-24]

The short-term effects described in this report, increased erythropoietin in the medium within 15 minutes after cAMP stimulation associated with a concomitant depletion of intracellular erythropoietin (Table 1), are consistent with secretion of preformed erythropoietin. The immediate release of an intracellular pool of preformed hormone in response to cAMP stimulation has been extensively reported in other endocrine systems. The present studies substantiate the utility of this human renal clear cell carcinoma cell line for investigation of cellular factors mediating the production of erythropoietin.

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Sardinian $\delta\beta^{\circ}$-Thalassemia: A Further Example of a C to T Substitution at Position −196 of the $^{A}\gamma$ Globin Gene Promoter

By Sergio Ottolenghi, Barbara Giglioni, Andrea Pulazzini, Paola Comi, Clara Camaschella, Anna Serra, Angelo Guerrasio, and Giuseppe Saglio

Selective overexpression (50- to 100-fold) in adult erythroid cells of either $^{G}\gamma$ or $^{A}\gamma$ fetal globin gene is observed in hereditary conditions known as $\delta\beta^{\circ}$-thalassemia and hereditary persistence of fetal hemoglobin (HPFH). Recently, a C → T change at position −196 of an overexpressed $^{A}\gamma$ globin gene from an Italian HPFH was hypothesized, on the basis of indirect evidence, to represent the cause of the functional defect. We now show that the same mutation is present in a different overexpressed $^{A}\gamma$-globin gene from a Sardinian patient with a different syndrome ($\delta\beta^{\circ}$-thalassemia). The Sardinian $^{A}\gamma$ globin gene differs from both the HPFH and the normal $^{A}\gamma$ globin gene at nucleotide 1,560 in the noncoding portion of the third exon, where an A is deleted. In addition, the mutant −196 $^{A}\gamma$-globin gene is linked to a normal β globin gene in HPFH, and to a β-thalassemic gene (β^{39}CAG → TAG) in $\delta\beta^{\circ}$-thalassemia. These data strengthen the suggestion that −196 mutation is causally linked to the abnormal phenotype and raise the question of whether the same or multiple mutational events are responsible for the appearance of the −196 mutation in different syndromes.

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THE MOLECULAR mechanisms regulating the coordinated and balanced activity of the human non-α globin genes (ϵ, $^{G}\gamma$, $^{A}\gamma$, δ, β)[1] during developmental switches (embryonic and fetal),[2,3] cell maturation,[4,5] and in different cell populations (A and F cells)[6,7] remain almost completely elusive, despite the enormous wealth of structural information accumulated in the last few years.[8,9] Some information has been provided, however, by genetic studies of disorders of the level of the expression of γ and β globin genes, which suggested the existence both of *trans*-acting factors[10-12] and of *cis*-acting loci whose deletion can influence γ or β globin gene activity.[8,9,13-25]

More recently, attention has focused on nondeletion syndromes characterized by increased expression of either the $^{G}\gamma$ or $^{A}\gamma$ globin genes during the adult period.[16,26-29] These conditions can be distinguished as hereditary persistence of fetal hemoglobin (HPFH) and $\delta\beta^{\circ}$-thalassemia on the basis of the greater clinical severity and of the unbalance of the α/non-α chain synthesis ratio in the latter. Recently, a correlation between γ globin gene overexpression and the occurrence of a single point mutation in one or two regions upstream of the cap site (within 200 nucleotides), has been found.[26-30]

These mutations might prevent the normal suppression of γ-globin gene transcription occurring after birth, although no direct proof for this hypothesis has yet been provided. Here we report that the same mutation originally observed in an overexpressed $^{A}\gamma$ globin gene from a patient with HPFH[27] is now found in an unrelated patient with Sardinian $\delta\beta^{\circ}$-thalassemia.

MATERIALS AND METHODS

DNA obtained from a Sardinian $\delta\beta^{\circ}$-thalassemic heterozygous patient[16] was digested with restriction enzymes and hybridized with cloned probes as described previously[10] to distinguish the γ globin cluster corresponding to the $\delta\beta^{\circ}$-thalassemic chromosome.

*Bgl*II-digested DNA was enriched in a 13-kilobase (kb) fragment containing both γ globin genes by centrifugation on a 5% to 20% NaCl gradient, and the resulting DNA was ligated into EMBL 4 *Bam*HI-digested arms and packaged in vitro. After screening, γ globin clones lacking the *Hind*III polymorphic sites ($\delta\beta^{\circ}$-thalassemic) were subcloned into pBR322, and sequencing was carried out according to Maxam and Gilbert.[31]

RESULTS

Sardinian $\delta\beta^{\circ}$-thalassemia is a syndrome characterized by selectively increased (50- to 100-fold) expression of the γ globin gene during adult life (with fetal hemoglobin—Hb F = $\alpha_2\gamma_2$—levels ranging between 10% and 20%) and by complete absence of β globin production by the β globin gene in *cis*.[16,32,33] This condition has been observed so far only in patients of Sardinian origin. To investigate the structure of the overexpressed $^{A}\gamma$ globin gene, we cloned a 13-kb *Bgl*II fragment (containing both the $^{G}\gamma$ and the $^{A}\gamma$ globin genes) from a heterozygous patient into phage. Family studies of restriction enzyme polymorphisms in Sardinian $\delta\beta^{\circ}$-thalassemic patients indicate that both their γ globin genes are characterized by the absence of a *Hind*III polymorphic site[34] in the second intron; this allowed us to distinguish, in the patient studied, the $\delta\beta^{\circ}$-thalassemic gene from the normal $^{A}\gamma$ globin gene. The cloned $^{A}\gamma$ globin gene was completely sequenced, from position −270 upstream of the cap site, to position +370 after the poly A addition site. Two major normal $^{A}\gamma$ globin gene sequences are known, corresponding to chromosomes A and B with different evolutionary histories (refs 35, 36, and Slightom, Shen, and Smithies, personal communication, June 1984) and showing numerous inser-

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tions, deletions, and single point mutations. The sequence of the thalassemic ${}^{A}\gamma$ globin gene closely matches that of the normal gene from chromosome B, with only two differences. The first mutation is a C-T substitution at position −196; the second mutation is the deletion of one nucleotide (A) at position 1,560 in the noncoding portion of the third exon (Fig 1); the same deletion is also found at the corresponding position of the ${}^{G}\gamma$ globin gene from chromosome B (Slightom, Shen, and Smithies, personal communication).

DISCUSSION

The C-T substitution at position −196 of the ${}^{A}\gamma$ globin gene which we detected in Sardinian $\delta\beta^{\circ}$-thalassemia may be responsible for the continued expression of this gene during adult life; alternatively, it may simply represent a polymorphic change with no relationship to the observed phenotype. We have previously described the same mutation in another overexpressed ${}^{A}\gamma$ globin gene that we detected in a Southern Italian patient with ${}^{A}\gamma$ HPFH.[27] More recently, Gelinas and colleagues[30] reported this mutation in an additional ${}^{A}\gamma$ globin gene from a Chinese patient with heterozygous ${}^{A}\gamma$ HPFH. On the other hand, in a collaborative study of HPFH and $\delta\beta^{\circ}$-thalassemia in Sardinia (with M. Pirastu, in preparation) the −196 mutation could be detected by oligonucleotide hybridization in $\delta\beta^{\circ}$-thalassemic patients only, but not in normal subjects, ruling out the hypothesis that this is a common polymorphism. Taken together, these results add further support to the idea that −196 mutation may be causally linked to ${}^{A}\gamma$ globin gene overexpression; it is of interest that two additional mutations, at position −202[26] and −198,[37] have been observed in overexpressed ${}^{G}\gamma$ and ${}^{A}\gamma$ globin genes, respectively, suggesting that a region around −200 may be relevant to γ globin gene regulation.

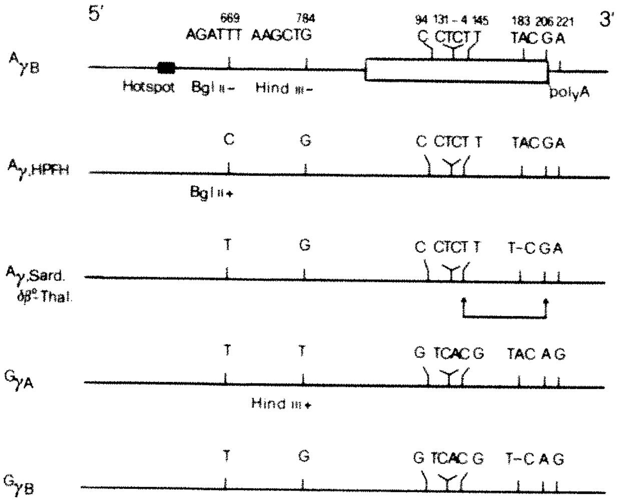


Fig 1. Nucleotide differences between Sardinian $\delta\beta^{\circ}$-thalassemia, Southern Italian hereditary persistence of fetal hemoglobin (HPFH), and normal γ globin genes. A normal γ globin gene is schematically represented. Numbers indicate nucleotide positions from the first nucleotide of the first exon and correspond to those given for the ${}^{A}\gamma$ globin gene from clone 165.24 in ref. 35. *BglII* and *HindIII* + or − indicate presence or absence of the corresponding restriction sites.[27,34] Nucleotide differences between the completely sequenced ${}^{A}\gamma$ globin genes from Southern Italian HPFH[27] and Sardinian $\delta\beta^{\circ}$ thalassemia (present article) and the normal ${}^{A}\gamma$ globin gene from chromosome B[35,36] are indicated. Also shown are nucleotide differences in the ${}^{G}\gamma$ (A and B) globin genes[35,36] *v* the normal ${}^{A}\gamma$ B globin gene; however, polymorphic differences 5′ to the *HindIII* site in the ${}^{G}\gamma$ globin genes are omitted for the sake of clarity. Gene conversion may have occurred between a ${}^{G}\gamma$ globin gene (type B) and the Sardinian $\delta\beta^{\circ}$-thalassemia ${}^{A}\gamma$ globin gene; arrows show limits of presumed event. The converted sequence cannot be longer than the distance (61 nucleotides) between positions 1,522 and 1,583 of the third exon, which are different in all ${}^{A}\gamma$ globin genes relative to ${}^{G}\gamma$ globin genes. C or G at position 1,471 is responsible for the insertion of alanine *v* glycine, respectively, in ${}^{A}\gamma$ and ${}^{G}\gamma$ globin chains.

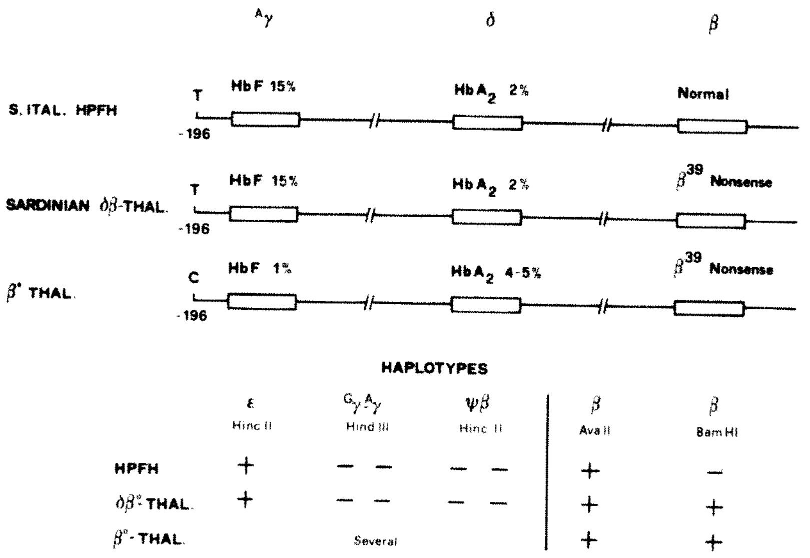
The observation of the same mutation in three different overexpressed ${}^{A}\gamma$ globin genes raises the question of single *v* multiple origin of the mutation. It has already been suggested that several mutations of the β globin gene (β^{S}, β^{E}, $\beta^{\circ 39}$) occurred more than once.[9] The Chinese −196 HPFH mutation[30] occurs in a ${}^{A}\gamma$ globin gene of type A, whereas the mutations in the two Italian cases are in a gene of type B that differs at several positions from the former.[35,36] It is therefore likely that the Mediterranean and Chinese mutations have independent origins.

It is less clear whether the two Italian mutations have the same or independent origins. The two genes differ at only two positions. The Southern Italian ${}^{A}\gamma$ gene has a T-C substitution at position 1,180 in the second intron, generating a polymorphic *BglII* site that is absent in both the normal and the $\delta\beta^{\circ}$-thalassemia ${}^{A}\gamma$ genes. The second difference is the loss of A at position 1,560 in the noncoding portion of the third exon that is observed in the $\delta\beta^{\circ}$-thalassemic but not in the Southern Italian HPFH ${}^{A}\gamma$ globin gene. This deletion may be the result of a rather complex event, possibly a limited interchromosomal or intrachromosomal gene conversion that transferred a polymorphic feature characteristic of the ${}^{G}\gamma$ globin gene type B to the ${}^{A}\gamma$ globin gene (Fig 1) as demonstrated for several other polymorphisms[35,36,38,39] These data are equally compatible with either a single origin for the Sardinian $\delta\beta^{\circ}$-thalassemia and Southern Italian HPFH −196 mutations (followed by the occurrence of private mutations in each of the two genes) or with two independent mutations at position −196 in different genes.

In addition, although the HPFH mutant ${}^{A}\gamma$ globin gene is linked to a structurally normal β globin gene,[27] expressed at an almost normal level, the $\delta\beta^{\circ}$-thalassemic ${}^{A}\gamma$ globin gene is linked to a β°-thalassemic gene[40,41] carrying a $\beta^{\circ 39}$ nonsense mutation[42] that is usually not associated with increased Hb F production. If we postulate a single origin of the −196 mutation (described above), a crossing-over event linking together this overexpressed ${}^{A}\gamma$ globin gene from an HPFH chromosome with a β-thalassemic gene must have occurred. The existence of a "hot spot" for crossing-over within a 9-kb region between the $\psi\beta$ and β globin genes has been previously suggested[9,43]; such a crossing-over would be compatible with the observed haplotypes (Fig 2).

Although we have no formal proof for this series of events, this hypothesis might be testable by looking for overexpressed −196 ${}^{A}\gamma$ globin genes unlinked to β°-thalassemia in the Sardinian population.

Remarkably, the Sardinian $\delta\beta^{\circ}$-thalassemia case is the first instance in which the joining of an overexpressed γ



	ε Hinc II	$^G\gamma\,^A\gamma$ Hind III	$\psi\beta$ Hinc II	β Ava II	β Bam HI
HPFH	+	− −	− −	+	−
$\delta\beta^\circ$-THAL.	+	− −	− −	+	+
β°-THAL.		Several		+	+

Fig 2. The $^A\gamma$-δ-β globin cluster in Southern Italian hereditary persistence of fetal hemoglobin (HPFH), Sardinian $\delta\beta^\circ$-thalassemia and β°-thalassemia (β^{39} nonsense mutation). Average Hb F and Hb A$_2$ percentages observed in heterozygotes are indicated. Several different γ globin genes (always producing low levels of γ globin) are linked to the β-thalassemic gene. All normal $^A\gamma$ globin genes sequenced so far have C at position −196, although direct data for $^A\gamma$-globin genes *cis* to β^{39} mutation are not available; however, preliminary hybridization experiments with specific oligonucleotides confirm this data. Restriction enzyme polymorphisms for β°-thalassemia (β^{39}-mutation) are from ref. 9 and M. Pirastu, personal communication. (Istituto di Clinica e Biologia dell 'ETA' Evolutiva, Caguari, Italy, September 1986).

globin to a β-thalassemic gene (whatever its mechanism) can be demonstrated to have significant physiological and possibly evolutionary effects; indeed, the high level of γ globin synthesis, by partially compensating for the decreased β globin synthesis, changes the severe β°-thalassemia phenotype (lethal in homozygous form) into a relatively mild condition that may be compatible with survival without transfusion in $\beta^\circ/\delta\beta^\circ$-thalassemic compound heterozygotes.[32] Considered in an evolutionary light, it is significant that although the overexpressed $^A\gamma$ globin gene of Southern Italian HPFH is very rare (only two families yet detected) in Southern Italy, the same gene in the $\delta\beta^\circ$-thalassemic context is relatively frequent in Sardinia (gene frequency: 0.1%) and represents a relatively high proportion (1.2%) of all β-thalassemic genes.[44] This fact suggests that the mutant $^A\gamma$ globin gene, because of its linkage to a β-thalassemic gene (that was under positive selection in this area) or because of the new phenotype (β-thalassemia with high fetal hemoglobin) may have conferred a selective advantage (relative to the normal and possibly also to the β-thalassemic population) to the carriers.

Heterozygous Sardinian $\delta\beta^\circ$-thalassemic patients exhibit, in addition to β°-thalassemia, normal or slightly decreased Hb A$_2$ levels, contrasting with the increased Hb A$_2$ level commonly observed in β-thalassemic heterozygotes. This finding may suggest the coexistence of a δ-thalassemic mutation; however, it has been observed that the expression of δ and β globin genes in *cis* to the −196 and −202 HPFH mutations is decreased, although direct determination of the nucleotide sequence of the β globin gene in Southern Italian $^A\gamma$ HPFH[27] and functional tests in $^G\gamma$ HPFH[26] indicate normality of the β globin genes. These data suggest that the decreased activity of β and δ globin genes in the latter syndromes (and of the δ globin gene in Sardinian $\delta\beta^\circ$-thalassemia) may be secondary effects somehow related to the hyperactivity of the mutated γ globin gene in *cis*.

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Development of T3/T Cell Receptor Gene Expression in Human Pre-T Neoplasms

By Stefania Pittaluga, Michael Uppenkamp, and Jeffrey Cossman

Acquisition of mature T cell function and the T cell antigen receptor repertoire occur in the thymus. In an effort to delineate the cascade of events leading to T cell maturation, we analyzed a series of clonal human precursor T cell neoplasms representing early, middle, and late stages of intrathymic differentiation. Rearrangements of the T cell receptor β and γ genes appear concurrently and are preceded by surface expression of the 3A1 (CD7) molecule. Subsequent transcription of the β gene is coordinated with surface expression of T1 (CD5) and T11 (CD2), transcription of T3δ mRNA, and the appearance of intracellular T3 (CD3) protein. As late events, Tα gene transcripts appear and, finally, T3, the multichain complex linked to the T cell receptor, is presented on the cell surface. Findings reported here provide a model of the developmental orchestration of genes encoding antigen recognition in human T cells.

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INTRATHYMIC T cell differentiation involves a multistep pathway featuring the rearrangement and transcriptional activation of genes encoding antigen recognition. Prior to expression of the cell surface membrane T cell antigen receptor (TCR),[1] genes encoding their α (T_α) and β (T_β) subunits must undergo successful variable region rearrangement, and their translational products combine with the multichain protein, T3.[1-11] Independent investigations of murine and human immature T cells implicate a hierarchy of events in which transcription of the T_β gene occurs early, persists throughout thymocyte development, and precedes the onset of T_α gene transcription.[12-18] Transcripts of a third rearranging gene, T_γ,[19-25] have been detected in fetal murine thymocytes, approximately coincident with the appearance of T_β mRNA, but, except for some murine cytotoxic T cells, remain for only a brief time.[17,18]

An ontogenic hierarchy of TCR α, β, and γ gene rearrangements analogous to immunoglobulin heavy, κ, and λ genes[26,27] might be suspected, but little information exists in this regard. Both T_γ and T_β genes rearrange early in thymic development,[18,21] but the ontogeny of T_α gene rearrangements has not yet been determined because murine and human J_α regions are too large (~35 kilobase [kb]) for conventional restriction fragment analysis.[6,28,29] With regard to T3, immunoreactive T3 protein appears to accumulate in the cytoplasm before surface expression when assayed in normal and neoplastic T cells.[30]

In addition to the development of antigen receptors, maturing thymocytes express a series of differentiation stage-related surface molecules, detectable with monoclonal antibodies, eg, T6 (CD1), T11 (CD2), T1 (CD5), and 3A1 (CD7) markers, which are useful for defining selected stages of early T cell differentiation.[31,32] Although the role of these cell surface molecules in T cell development is not known, some cell surface molecules apparently are coordinately expressed with the TCR-T3 developmental cascade. For example, cell surface expression of 3A1 (CD7) can be found in neoplastic precursor T cells prior to T_β gene rearrangement,[33] whereas other T cell-associated surface molecules generally appear only after T_β rearrangements occur.[33,34]

To determine the sequence of rearrangement and transcription of the TCR_α, β, and γ genes in humans, we investigated a series of human precursor T cell neoplasms representing early, middle, and late stages of thymocyte differentiation. These studies revealed a hierarchy of rearrangement and expression of TCR genes synchronous with changing surface marker profiles and expression of the T3 complex. Analysis of pre-T neoplasms suggests a model of successively expressed genes coordinated with development of the TCR mechanism.

MATERIALS AND METHODS

Cells. T lymphoblastic neoplasms were classified by conventional microscopy as either acute lymphoblastic leukemia or lymphoblastic lymphoma and immunotyped as T cell lineage as described.[33] Viable cell suspensions were used for cell surface marker study and DNA/RNA extraction. T acute lymphoblastic leukemia cell lines, provided by the American Type Culture Collection (Rockville, MD) included CEM, 8402, Molt-4, and Jurkat.

Cell surface phenotyping. Viable cells were treated with monoclonal antibodies and analyzed by indirect immunofluorescence in a fluorescence-activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, CA) as described.[35] Cytoplasmic staining of T3 was performed on acetone-fixed cytocentrifuge preparations with an avidin–biotin complex immunoperoxidase detection technique as previously outlined.[35]

DNA/RNA analysis. High-molecular-weight DNA was extracted from lymphoid cells and digested to completion with the appropriate restriction endonuclease (*Bam*HI, *Eco*RI, *Hind*III, and *Bcl*-I from Biolab, New England Nuclear, Beverly, MA), size-fractionated by electrophoresis in agarose gels, and transferred to nylon paper (Gene Screen Plus, Dupont, New England Nuclear).[33] The blots were hybridized with random-primed ^{32}P-labeled DNA probes at specific activities of 300 to 600 cpm/pg. The following probes were used: a subcloned constant region of the β chain (*Ava*I-*Pst*I fragment of cDNA YT35 provided by T. W. Mak[2]), a full-length cDNA of T_α (PY14 provided by T. W. Mak[6]), and a genomic fragment including the J_1 region of the human T_γ gene (*Hind*III-*Eco*RI fragment of M13H60 provided by T. Rabbitts.[22,24]) Blots were autoradiographed following high-stringency washes.

Total cellular RNA was isolated by ultracentrifugation on a guanidinium isothiocyanate/$CsCl_2$ gradient.[36] Twenty micrograms

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of total RNA was subjected to electrophoresis through 1% agarose/formaldehyde denaturing gels and was transferred to nylon filters. Hybridization was carried out with probes described above and also with a human $T3_\delta$ cDNA[37] provided by C. Terhorst, Harvard Medical School. High-stringency washes were carried out at 50°C in 0.1X SSC for 15 minutes, and blots were autoradiographed by standard techniques.

RESULTS

Phenotyping. All samples were unreactive with all B cell markers and expressed one or more T cell markers (Table 1). Each strongly reacted with 3A1 (CD7), but other T cell markers were selectively expressed in these early T cell neoplasms. For example, T11 (CD2) was negative in 2 cases (1 and 3) which alternatively expressed T1 (CD5); T6 (CD1), a marker expressed by cortical thymocytes, was unreactive in 5 of 10 samples (1, 2, CEM, 8402, HSB); and surface T3 (CD3) was positive in only 2 of 10 samples. In contrast, intracellular T3 was detectable in all but 3 cases (1, 2, and 3) and displayed a combined nuclear membrane and cytoplasmic distribution (Fig 1).

DNA rearrangements. T_β gene rearrangements were demonstrated in three of five primary cases and in all cell lines. Cases 1 and 2 displayed only germline configurations of the T_β genes when analyzed with three different restriction enzymes (*Bam*H1, *Eco*RI, and *Hind*III), as previously reported.[33] T_β gene rearrangements involved a single allele (cases 3, 5, and Jurkat) or both alleles (in all cell lines) and, in one exceptional situation, three nongermline T_β bands and deletion of the germline band (case 4). This latter feature was confirmed by analysis with all three restriction endonucleases. By cytogenetic analysis, case 4 exhibited trisomy of chromosome 7, which carries the T_β locus at band 7q35[38-40]; thus, the three nongermline bands are most likely a consequence of rearrangements of each T_β gene in this cell.

Rearrangements of the T_γ gene were detected in all cases and cell lines, except for cases 1 and 2 (Table 1, Fig 2). Thus, the presence or absence of T_γ and T_β gene rearrangements coincided in each sample tested. Case 4, which harbored a trisomy of chromosome 7, showed three different rearrangements of the T_γ gene, as it did for T_β. Presumably, the three nongermline bands are due to three copies of the T_γ gene, located at chromosome 7p15.[23]

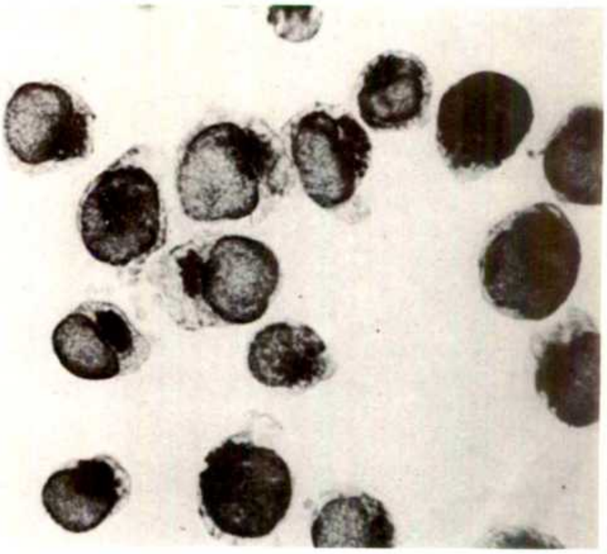

Fig 1. Intracellular T3 (cT3). Cytocentrifuge preparations were immunostained by an avidin-biotin peroxidase complex detection system using Leu4 as the primary antibody. In this example Jurkat cells display dense, intracytoplasmic, perinuclear reaction product. In many cells the nuclear membrane also appears to be stained. (Original magnification, ×600; current magnification ×570.)

T_α gene rearrangements were undetected in many cases, including cases 1 and 2, despite digestion with several restriction enzymes (Fig 3). Lack of nongermline bands might be due to either unrearranged T_α genes or to the unusually large size of the T_α genome.[6,28,29] We were able to identify a T_α rearrangement in Jurkat and a deletion of a T_α band in Molt-4.

mRNA transcription. T_β mRNA was detectable in each sample (Fig 4) except cases 1 and 2, which contained germline T_β genes. Case 3, with an early phenotype (3A1+, T11−, T1+) but a rearranged T_β gene, showed only the incomplete or "immature" 1.0-kb T_β message, presumably transcribed from a C_β gene that had not combined to variable

Table 1. Phenotyping of Samples

Cells	3A1 (CD7)	T1 (CD5)	T11 (CD2)	T6 (CD1)	cT3 (CD3)	sT3 (CD3)	T_β		T_α		T_γ	$T3_\delta$
							DNA	RNA	DNA	RNA	DNA	RNA
Case 1	+	+	−	−	−	−	G	—	G	—	G	—
Case 2	+	−	+	−	−	−	G	—	G	—	G	—
Case 3	+	+	−	+	−	−	R	1.0	G	—	R	0.7
Case 4	+	−	+	+	+	−	RRR	1.3	G	—	RRR	0.7
Case 5	+	+	+	+	+	+	R	1.0, 1.3	G	1.3, 1.5	RR	0.7
CEM	+	+	+	−	+	−	RR	1.3	G	—	RR	0.7
8402	+	+	+	−	+	−	RR	1.3	G	—	RR	0.7
HSB	+	+	+	−	+	−	RR	1.3	G	—	RR	0.7
Molt-4	+	+	+	+	+	−	RR	1.3	GD	—	RR	0.7
Jurkat	+	+	+	+	+	+	RR	1.0, 1.3	R	1.3, 1.5	RR	0.7

+, >50% of cells stained; −, <20% of cells stained; cT3, intracellular T3; sT3, surface T3; G, germline; R, rearranged; D, deleted; —, none detected; mRNA sizes are given in kilobases.

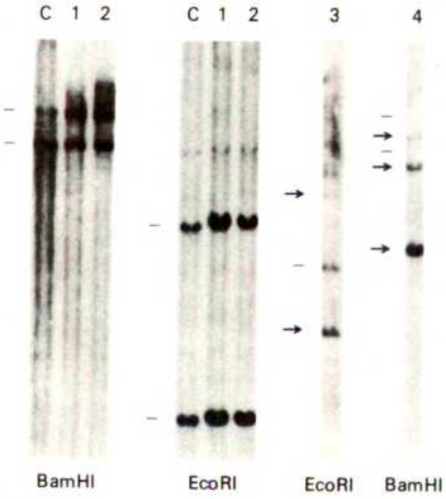

Fig 2. T$_\gamma$ gene rearrangements. Genomic DNA was digested with the indicated enzyme, size separated in agarose gels, and analyzed on Southern blots with the J$_\gamma$ probe. Lanes are numbered according to the case number, C represents a germline (unrearranged) control, and the germline bands are indicated with dashes adjacent to the gel. No rearrangements were detected in either case 1 or 2 when digested with both enzymes *BamHI* or *EcoRI*. Rearrangements were detected in all other cases; for example, two rearrangements were seen in an *EcoRI* digest of case 3, and three rearrangements were present in a *BamHI* digest of case 4.

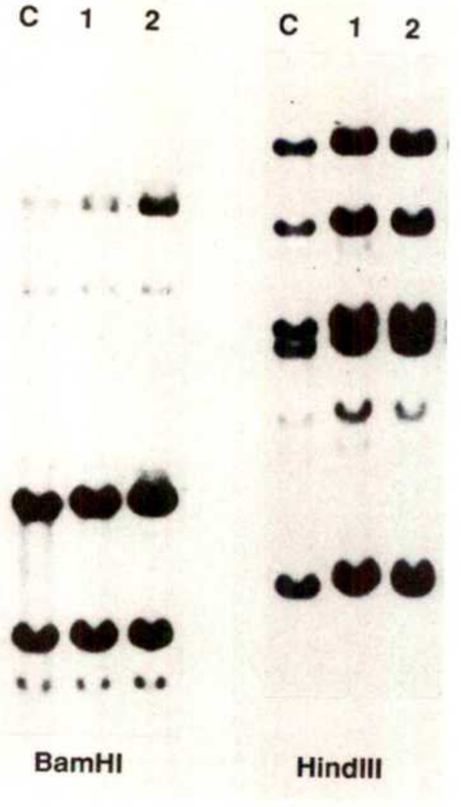

Fig 3. Southern blot analysis of the T$_\alpha$ gene. Despite digestion with two different restriction enzymes, *BamHI* and *HindIII*, both cases 1 and 2 showed the same restriction fragment as the germline control (C).

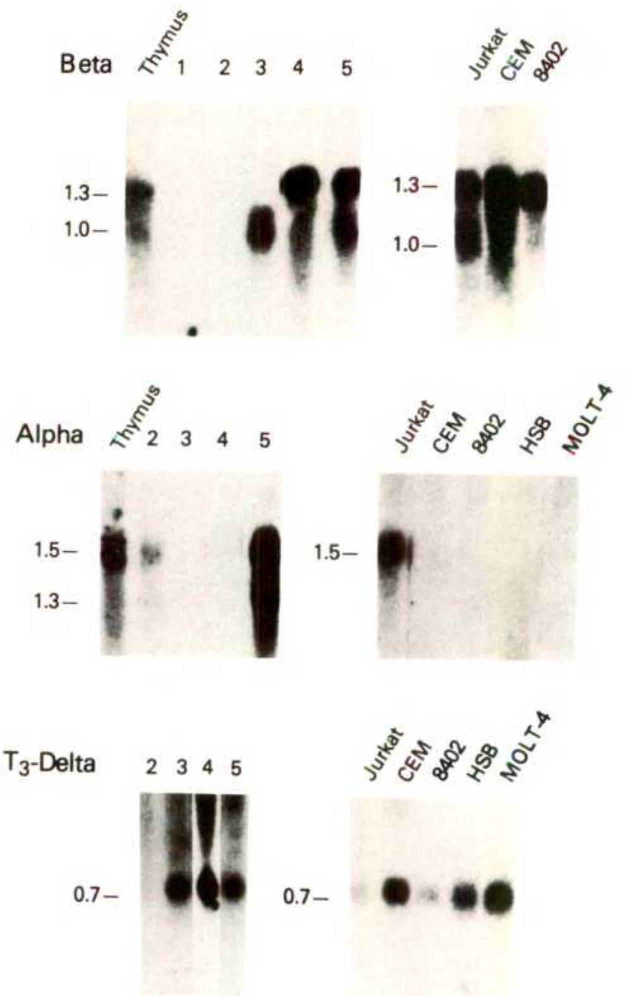

Fig 4. Northern blot analysis of β, α, and T3δ mRNA. Lane numbers refer to the individual case numbers, and mRNA sizes are given in kilobases (kb) on left. β Transcripts were detected in all samples except cases 1 and 2. Both the 1.3-kb and 1.0-kb message sizes could be detected in several cases. T$_\beta$ transcripts were detected in normal thymus, case 5, and in the Jurkat cell line. No transcripts were seen in either cases 3 or 4, and the extremely faint band in case 2 appearing after prolonged exposure is most likely attributable to a small percentage of normal T cells (~7%) contaminating this sample. T3δ mRNA was found in varying abundancy in most cases but was not detected in cases 1 or 2.

sequences.[41] Both mature (1.3 kb) and immature (1.0 kb) T$_\beta$ messages were simultaneously present in case 5 and in Jurkat.

T$_\alpha$ mRNA was detectable only in case 5 and in Jurkat and, in each of these leukemias, both the incomplete (1.3 kb) and mature (1.5 kb) messages were transcribed (Fig 4). These two T cell neoplasms containing T$_\alpha$ transcripts, case 5 and Jurkat, were also the only cells with surface T3 expression. T$_\gamma$ mRNA was not detected in any samples tested.

T3δ transcripts 700 base pairs (bp) in length were detected in all samples except cases 1 and 2, which were also the same leukemias devoid of detectable intracellular and surface T3 protein (Fig 4). Intracellular T3 protein and T3 mRNA were simultaneously present in all other samples.

DISCUSSION

T lymphoblastic leukemias and lymphomas are relatively uniform clones considered to be arrested at intrathymic stages of differentiation; they generally reflect the phenotypically characterized compartments of early T cell ontogeny.[31] Together, lymphoblastic malignancies of T and B cell lineage represent neoplasms of lymphocyte precursors and thereby serve as models for the evaluation of lymphocyte development.[12-14,26,30,31,33-35,42] The understanding of the development of immature thymocytes into mature, functional T cells has been expanded by analysis of precursor T cell neoplasms obtained from either patient tissue[30,31,33,34] or cell lines.[12-14,30,31] Direct comparisons of neoplastic cells to normal cell differentiation, however, must take into consideration properties of the malignant cells, such as aberrant gene expression and potential instability of phenotype during cell culture. In the present study, we analyzed primary cases and well-characterized cell lines which, collectively, span the phenotypic range of early T cell development.

Precursor T cell neoplasms presented here encompass a spectrum of intrathymic T cell development ranging from primitive cells, whose antigen receptor genes were retained in the germline configuration, to cells with rearranged and transcribed antigen receptor genes and a nearly mature T cell phenotype. Associated with developments in antigen receptor gene recombination and expression was the sequential appearance of T cell-associated markers. By analyzing each of these critical features in individual precursor T cell clones, we have begun to piece together the coordination of events in the T cell developmental process.

The earliest identifiable stage, analogous to the murine "prothymocyte,"[15] was surface expression of 3A1 (CD7), in association with either T1 or T11, in cells whose TCR genes were retained in the germline form. Neither T_β[33] nor T_γ gene rearrangements were demonstrable at this stage (cases 1 and 2) despite digestion with several restriction enzymes, and neither gene was apparently transcribed. Lack of nongermline T_α genes in these cases does not preclude the presence of T_α rearrangements because of the difficulty inherent in detection of gene rearrangements at this locus.[6,28,29] T_α gene rearrangement, however, in the absence of rearrangement of T_β and T_γ genes, is unprecedented, and absence of T_α mRNA suggests that T_α genes might not yet be rearranged in cases 1 and 2. Further indication of immaturity in cases 1 and 2 are the lack of expression of T6, the cortical thymocyte marker, and the absence of intracellular as well as surface T3. Alternatively, it could be argued that such primitive cells are not committed to T cell differentiation. These lymphoid cells show no evidence of B cell commitment, however, since they lack B cell markers,[33] have germline immunoglobulin genes,[33] and the 3A1+, T1+, and 3A1+, T11+ phenotypes are not found in B lineage neoplasms.[26,42]

T_β rearrangement occurs early in thymocyte differentiation and most precursor T cell neoplasms have similarly undergone such rearrangements.[43-46] Rearrangements may involve one or both alleles and, in one exceptional case reported here, trisomy of chromosome 7 apparently provided for rearrangement of three T_β and three T_γ alleles. In early stages, T_β transcripts are incomplete, and their 1.0-kb size results from a D-J-C transcript following D-J joining.[41] Case 3 in the current study is characterized by transcription of the 1.0-kb T_β mRNA without detectable complete (1.3 kb) transcripts. Its early thymocyte phenotype, 3A1+, T11−, T1+, T6+, cT3−, sT3−, and lack of T_α transcripts are indicative of an early differentiation stage. As with T_β, it is in this cell that we see the first evidence of T_γ gene rearrangement. Whether T_β and T_γ genes rearrange concurrently in human cells cannot be ascertained by this analysis but, based on findings reported here, rearrangements of both genes apparently occur as early events in T cell differentiation. It is noteworthy that T_β and T_γ genes were always simultaneously rearranged.

As a subsequent step, full-sized 1.3-kb T_β mRNA was detected. The "mature" T_β message was seen in all five cell lines and in cases 4 and 5. Provocatively, intracellular T3 was identified only when the 1.3-kb T_β mRNA was detected, a finding that suggests the possible existence of a common factor(s) that might regulate the coordinated expression of T_β and T3 genes. Intracellular T3 is known to appear early in thymocyte differentiation, prior to localization on the outer plasma membrane.[30]

Surface expression of T3 was identified only in cases in which T_α mRNA was detected. Two sizes of T_α mRNA, 1.5 and 1.3 kb, which likely represent complete and incomplete messages, respectively,[4] were detectable in both case 5 and Jurkat. Lack of expression of surface T3 in the absence of T_α transcription is consistent with obligatory production of a complete α-β heterodimer, which is noncovalently linked to the T3 complex for surface membrane T3 to appear.[8,11] Although T_γ mRNA was not observed in any of the leukemias analyzed, low abundancy message might not be demonstrable by use of a genomic J_γ probe. Further analysis with cDNA probes will be necessary to determine whether the T_γ

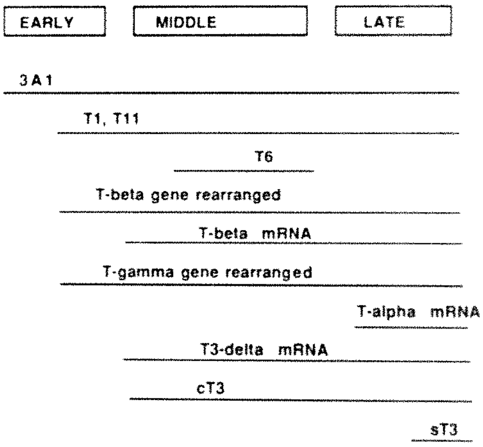

Fig 5. Hierarchy of T3/T cell receptor development. Precursor T cell neoplasms can be roughly segregated into three compartments—early, middle, and late. The phenotypic expression of cells within each compartment is shown along with the chronology of events beginning with rearrangement of T cell receptor genes and progressing to their transcription and, ultimately, to expression of the T3 molecular complex.

gene is transcriptionally active in these human pre-T neoplasms.

The picture that has emerged from the current analysis of precursor T cell neoplasms is a cascade beginning with 3A1 expression, followed by T1 and T11 expression, and T_β and T_γ gene rearrangement; T_β gene transcription, $T3_\delta$ gene transcription, and intracellular T3 accumulation; and, finally, T_α transcription and the appearance of surface T3 (Fig 5). In all, the elaboration of the antigen recognition function of T cells is shaped by regulated events within the thymus. It is tempting to speculate that regulation of antigen receptor development is mediated by surface molecules, such as 3A1, T1, and T11, which might serve as receptors for humoral or cellular factors present in the thymic microenvironment. In this regard, it is interesting to note that 3A1 appears on precursor cells, "prothymocytes," prior to entry into the thymus, and perithymic mesenchyme expresses a moiety detectable with antibody 3A1.[47] Analysis of the coordination of gene expression during T cell differentiation should provide leads into the identification of regulatory molecules.

ADDENDUM

The ordered hierarchy of rearrangement and expression of genes of the T cell receptor–T3 complex outlined in the present investigation is consistent with recently reported studies of murine fetal thymocytes[48] and human T cell leukemias.[49]

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Identification of a Functional Role for Human Erythrocyte Sialoglycoproteins β and γ

By Marion E. Reid, Joel Anne Chasis, and Narla Mohandas

Four distinct erythrocyte membrane sialoglycoproteins (SGPs) denoted α, β, γ, and δ have been described, but their functions have not yet been defined. Recent evidence suggests that several of these SGPs associate with membrane skeletal proteins. Because the membrane skeletal protein network plays an important role in regulating the membrane material properties of deformability and mechanical stability, we wanted to determine whether the SGPs, through their interaction with the membrane skeleton, can modulate these membrane properties. We measured membrane mechanical stability and membrane deformability of erythrocytes that were deficient in either α, or δ or β and γ SGPs. Only erythrocytes deficient in β and γ SGP had altered membrane properties, as evidenced by marked decreases in both membrane mechanical stability (50% of normal) and membrane deformability (40% of normal). Erythrocytes deficient in either α or δ SGP had normal deformability and stability. Based on these data, we suggest that an interaction of β and/or γ SGP with the membrane skeleton plays a functionally important role in regulating normal erythrocyte membrane properties.

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FOUR DISTINCT erythrocyte membrane sialoglycoproteins (SGPs) denoted α, β, γ and δ carrying different blood group specificities have been described,[1] but their role in regulating membrane function has not been defined. Recent evidence suggests that several of these SGPs associate with membrane skeletal proteins.[2-9] More specifically, both α and β SGP[5] have been shown to interact with protein 4.1, and the interaction between α SGP and protein 4.1 may be modulated by phosphoinositides.[3] Because the membrane skeletal protein network plays an important role in regulating the membrane material properties of deformability and stability, we wanted to determine whether SGPs, through their interaction with the membrane skeleton, can modulate these membrane properties. We measured mechanical stability and membrane deformability of erythrocytes that were deficient in either α or δ or β and γ SGPs. We found that only erythrocytes deficient in β and γ SGPs exhibited decreased mechanical stability and deformability, whereas erythrocytes deficient in either α or δ SGP had normal membrane mechanical properties. These data allow us to define a functionally important role for β and γ SGPs, that of regulating membrane properties of deformability and stability, possibly through their interaction with components of the skeletal protein network. The data further raise the possibility that membrane properties of other types of cells may be modulated by similar associations between cytoplasmic domains of transmembrane proteins and cytoskeletal elements.

MATERIALS AND METHODS

After informed consent was obtained from subjects with various SGP protein deficiencies, blood samples were drawn into acid citrate dextrose and analyzed within 30 hours of collection. SGP-deficient samples tested were from the following patients: E.P. for the α SGP deficiency, A.T. and R.W. for δ SGP deficiency and P.L., J.C., S.S., and T. L. for the β and γ SGP deficiencies.

Immunochemical and biochemical analysis. The absence of specific SGP was confirmed by immunochemical analysis of erythrocyte membranes. Preparation of erythrocyte membranes, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), periodic-acid Schiff's base (PAS) staining and immunoblotting were performed as described previously.[6,10] The monoclonal antibodies used for immunoblotting have been reported in detail.[6,11] In brief, LICR/R1.3 (R1.3) detects an epitope carried on the N terminus of both α and δ SGPs,[11] and NBTS/BRIC-10 (BRIC-10) recognizes an epitope on the N terminus of β SGP.[6] The polyacrylamide concentration was 3% for the stacking gel and 10% for the separating gel for PAS staining and Western immunoblotting and 3.5% to 17% nonlinear gradient gel for Coomassie brilliant blue staining.

The relative concentration of various membrane proteins was determined by the pyridine elution method previously described.[12] After destaining of Coomassie brilliant blue-stained SDS-PAGE gels, appropriate areas of gel corresponding to spectrin, band 3, protein 4.1, and actin were cut; the stain was eluted with pyridine and quantitated by measurement of absorbance at 605 nm. The relative concentration of skeletal protein was quantitated by determining the absorbance ratios of spectrin, protein 4.1, and actin to band 3.

To assess the state of spectrin self-association, nondenaturing PAGE was performed on spectrin extracted from normal and SGP-deficient membranes.[13,14] Spectrin was extracted by dialysis against hypotonic buffer (0.3 mmol/L of Na phosphate, 0.1 mmol/L of EDTA and 0.1 mmol/L of phenylmethylsulfonyl fluoride (PMSF) pH 8.0) at 4°C for 17 hours. The percentage of spectrin dimer (expressed as dimer/dimer plus tetramer) was determined by densitometric analysis of the nondenaturing gels.

Membrane deformability and mechanical stability measurements. Resealed membranes for deformability and stability measurements were prepared by a procedure adopted from Johnson.[15] Erythrocytes were washed three times in 5 mmol/L of Tris and 140 mmol/L of NaCl (pH 7.4). The erythrocytes were then lysed in 40 vol of 7 mmol/L of NaCl and 5 mmol/L of Tris (pH 7.4). The membranes were pelleted by centrifugation, resuspended in 10 vol of 5 mmol/L of Tris and 140 mmol/L of NaCl (pH 7.4), and incubated for 60 minutes at 37°C to promote resealing.

To measure membrane stability, 100 μL of resealed membranes pelleted by centrifugation was mixed with 3 mL of dextran (40,000

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mol wt, 35 g/100 mL in 10 mmol/L of phosphate buffer, viscosity 95 centipoise, pH 7.4), and subjected to a continuous applied shear stress of 750 dynes/cm^2 in the ektacytometer.[16] Under this stress, the membranes progressively fragment, generating nondeformable spherical fragments. This process is detected as a time-dependent decrease in the deformability index (DI). The time required for the DI to fall to 50% of its maximum values is termed T_{50} and is a quantitative measure of membrane stability.[16]

The property of membrane deformability that determines the extent of membrane deformation that can be induced by a defined level of applied force was also measured by ektacytometry. For resealed membranes, the shear stress required to obtain a defined value of DI is determined by the property of membrane deformability, without contributions from either internal viscosity or cell volume.[17] A correlation exists between changes in deformability measured by this technique and those measured using the micropipette.[17] To measure this property, 30 μL of resealed ghosts were suspended in 3 mL of Stractan (22 cp viscosity, 290 mOsmol/kg, pH 7.4). The ghosts were then subjected to a linearly increasing shear stress in the ektacytometer. The DI was measured as a function of applied shear stress. When DI is plotted on a linear scale and the shear stress is plotted on a log scale, a linear relationship exists between these two variables for normal membranes. For membranes with decreased deformability, the linear relationship still exists; however, there is a parallel displacement of the lines to higher values of shear stress. From the differences in shear stress values required to obtain equivalent DIs, a value for relative membrane deformability is derived.[17,18]

RESULTS

Erythrocytes deficient in α or δ or β and γ SGPs were obtained from individuals who had previously been identified as lacking these SGPs.[6,19-21] The absence of specific SGP was confirmed by immunochemical analysis on erythrocytes from these donors. Figure 1 shows a representative example of each type of SGP deficiency. Coomassie brilliant blue staining of SDS polyacrylamide gradient gels showed that membranes deficient in α or δ or β and γ had the normal complement of skeletal proteins. PAS staining showed that membranes from E.P. were deficient in α SGP, membranes from A.T. were deficient in δ, and membranes from J.C. were deficient in β. Immunoblotting with R 1.3 (a monoclonal antibody that reacts with α and δ SGPs) showed that α and δ SGP-deficient erythrocytes lacked all bands corresponding to these SGPs, whereas control and β and γ-deficient erythroyctes had a normal complement of α and δ SGPs. Immunoblotting with another monoclonal antibody, BRIC-10 (an antibody with specificity for β SGP) showed that β SGP was absent only in β- and γ-deficient membranes and was present in normal amounts in control, α-deficient, and δ-deficient erythrocytes.

The two key, but distinct, membrane material properties, mechanical stability and deformability, are regulated by the skeletal proteins spectrin, actin, and protein 4.1 and their interactions.[12,22-28] Qualitative defects of spectrin and quantitative deficiencies of spectrin or protein 4.1 content were previously shown to result in marked decreases in membrane mechanical stability.[16] To define if deficiencies of various SGPs can also lead to altered membrane properties, the deformability and stability of resealed ghosts made from the SGP-deficient erythrocytes were measured using an ektacytometer.[16-18] In this system, resealed ghosts are exposed to a constant value of applied shear stress. The rate at which the deformability of resealed membranes decreases as a result of membrane fragmentation is used to quantitate membrane stability (Fig 2). The decrease in DI as a function of time for resealed membranes prepared from erythrocytes lacking either α or δ SGP was the same as that for control membranes, implying that these membranes had normal mechanical stability. On the other hand, membranes prepared from erythrocytes from all four individuals lacking β and γ SGPs fragmented at a much faster rate than normal

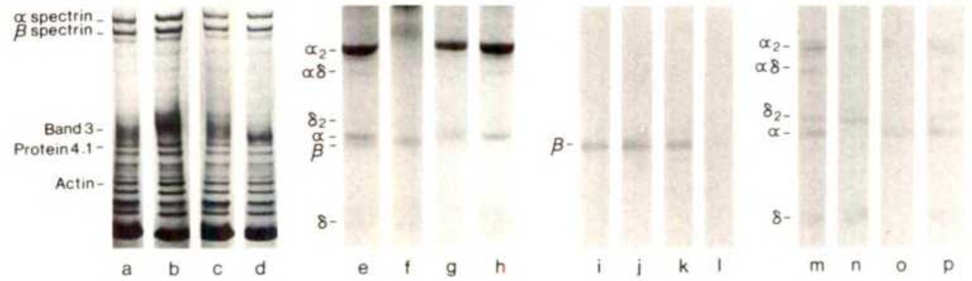

Fig 1. Immunochemical analysis of erythrocyte membranes. Coomassie brilliant blue-stained (lanes a through d), periodic-acid Schiff (PAS)-stained (lanes e through h), and Western blots (lanes i through p) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of sialoglycoprotein (SGP)-deficient membranes: results from a representative example of each type of SGP deficiency. Coomassie brilliant blue staining of SDS-polyacrylamide gradient gels showed that membranes deficient in α (lane b), δ (lane c), or β (lane d) had the normal complement (lane a) of skeletal proteins. The only observed difference between the various samples and control membranes was the decreased mobility of band 3 in the α-deficient membranes. This phenomenon has been described previously and is due to an increase in size of the *N*-glycosidically linked oligosaccharide on band 3.[41] The SGP components of these membrane preparations was determined following staining with PAS (lanes e through h). Membranes from E.P. were deficient in α SGP (lane f), membranes from A.T. were deficient in δ SGP (lane g), and membranes from J.C. were deficient in β SGP (lane h). The PAS-stained gel also illustrates the increased glycosylation of band 3 in the α-deficient membranes. Immunoblotting with antibody BRIC-10 showed that β SGP is absent only in β-deficient membranes (lane l). Normal complement of β SGP is present in control membranes (lane i) and membranes from erythrocytes lacking α or δ SGP (lanes j and k). Immunoblotting with the R1.3 shows that α SGP-deficient erythrocytes lack bands corresponding to monomeric α, dimeric α, and the heterodimer of α and δ (lane n). Membranes from erythrocytes deficient in δ SGP lack monomeric δ, dimeric δ, and the heterodimer of α and δ (lane o). The α and δ SGP content was normal in control erythrocytes (lane m) and in the β-deficient erythrocytes (lane p).

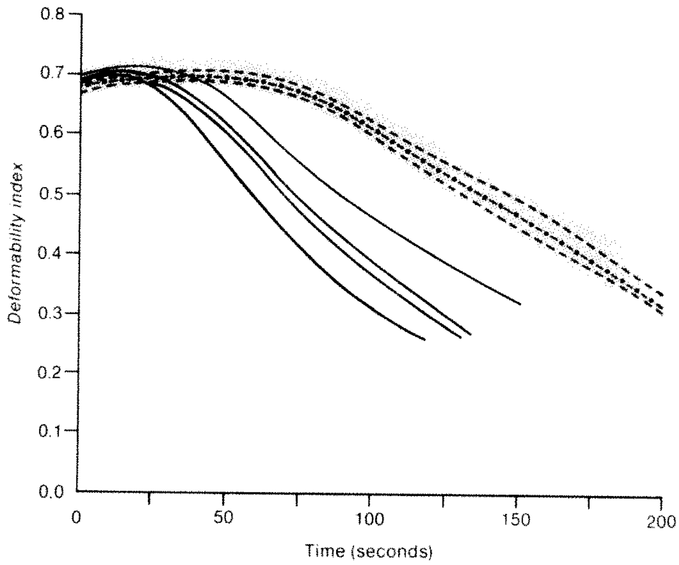

Fig 2. Membrane stability of sialoglycoprotein (SGP)-deficient erythrocytes. The deformability index of erythrocyte membranes deficient in β and γ SGPs decreased more rapidly with time when exposed to constant shear stress (—) when compared with normal erythrocyte membranes (shaded area), implying decreased mechanical stability. In contrast, the rate of decline of deformability index of erythrocyte membranes deficient in α SGP (- - -) and δ SGP (---) was normal, implying that these membranes had normal mechanical stability. α SGP-deficient membranes were tested on three different occasions and yielded similar results.

membranes. The mechanical stability of these membranes was only 50% of normal (range 42% to 62%).

The property of membrane deformability determines the extent of membrane deformation that can be induced by a defined level of applied force. A value for relative membrane deformability of erythrocytes is derived by comparing the values of applied shear stress required in the ektacytometer to obtain equivalent membrane deformation for various resealed ghost preparations. As shown in Fig 3, membranes that lacked α or δ SGP underwent the same extent of membrane deformation as did control membranes at equivalent values of applied shear stress. This implies that in addition to having normal mechanical stability, these membranes were also normally deformable. In contrast, membranes lacking β and γ SGPs required higher levels of applied shear stress than did control membranes to reach equivalent membrane deformation, implying that these membranes have decreased membrane deformability. The membrane deformability of β-deficient and γ SGP-deficient erythrocytes from the four individuals was, on the average, only 40% of normal (range 27% to 46%).

In addition to these alterations in membrane properties, the β and γ SGP-deficient erythrocytes were elliptocytic, an abnormality that has been described previously.[6,21] In con-

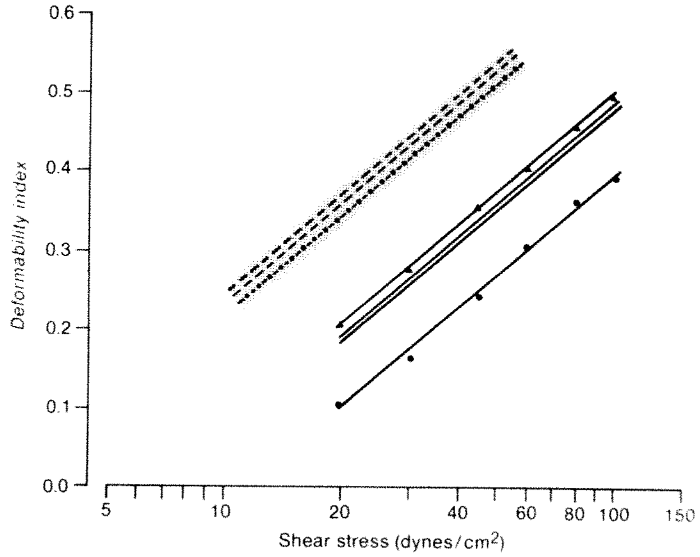

Fig 3. Membrane deformability of sialoglycoprotein (SGP)-deficient erythrocytes. Erythrocyte membranes deficient in β and γ SGPs required significantly higher values of applied shear stress (—) as compared with normal membranes (shaded area) to reach equivalent deformation. The lines are parallel; thus, β and γ SGP-deficient membranes required 2.5-fold higher shear stress than did normal membranes to reach equivalent deformation at all points along the curve, indicating that the membranes had 0.4 times normal deformability. In contrast, α SGP-deficient (-.-) and δ SGP-deficient (---) membranes required the same shear stress as normal membranes to reach equivalent deformation, implying normal membrane deformability. α SGP-deficient membranes were tested on three different occasions and yielded similar results.

trast, erythrocytes lacking α or δ or both α and δ had normal discoid morphology.[29,30] Decreased membrane stability and deformability in conjunction with elliptocytic morphology have previously been documented in hereditary elliptocytosis as a consequence of primary skeletal protein defects. In these cases, defective oligomerization of spectrin resulting from qualitative defects in α-spectrin, as well as protein 4.1 deficiency have been identified as responsible for the membrane abnormalities.[26,27,31,32] To rule out the possibility that similar skeletal protein abnormalities were involved in the altered properties of the β and γ SGP-deficient erythrocytes, we measured the spectrin and protein 4.1 content of these membranes as well as the state of spectrin oligomerization in the membrane. The ratios of both spectrin and protein 4.1 to band 3 were normal (spectrin to band 3, 0.95-1.05, and protein 4.1 to band 3, 0.9-1.05). In addition, the ratio of spectrin dimers to dimers plus tetramers in the membranes from two unrelated β and γ SGP-deficient individuals was within normal limits (P.L. 3.5%, S.S. 4.4%, normal range 2.0% to 5.4%), a result consistent with normal spectrin self-association.[13,14] Thus, the membrane and morphological abnormalities we describe are primarily due to the SGP deficiency and not to any of the skeletal protein alterations previously identified.

DISCUSSION

The data from the present study allow us to define an important functional role for SGP β and γ in erythrocytes, that of regulating membrane physical properties. Because deformability and stability are largely determined by the membrane skeleton, the data further imply that regulation of membrane properties by SGP β and γ may occur through the interaction of these SGPs with the membrane skeletal network. Experimental evidence to support such an interaction has been previously reported by several investigators[4,5,7,8] and, more specifically, it appears that SGP β interacts with the protein 4.1 component of the membrane skeleton.[4,5] In vitro studies have also shown interaction between SGP α and protein 4.1,[2,3,9] but this interaction does not appear to regulate either membrane deformability or stability since erythrocytes deficient in this major SGP have normal membrane properties. This raises the possibility that although SGP α has the potential to interact with protein 4.1, this interaction may not occur in normal erythrocyte membranes. Support for such a suggestion comes from recent experimental evidence which has shown that although SGP α can be removed readily and totally from normal membranes by detergent extraction, binding of specific ligands to SGP α induces the association of this SGP with the membrane skeleton and a concomitant decrease in membrane deformability.[33] Thus, it appears that while SGP β is normally associated with the membrane skeleton and plays a physiologically important role in regulating membrane properties, the interaction of SGP α with skeletal proteins is regulated by ligand binding or by changes in phospholipid metabolism.[3,33]

β and γ SGP-deficient erythroyctes were previously reported to be resistant to invasion by *Plasmodium falciparum*.[34] The present data suggest that the decreased membrane deformability that we identified may play a role in this resistance. Precedence for this suggestion is indicated by the close relationship between decreased membrane deformability and decreased malarial parasite invasion previously documented in ovalocytes.[35,36]

The state of spectrin self-association and spectrin–actin–protein 4.1 interaction were previously shown to play a key role in regulating membrane mechanical stability.[26,27,31,32] Decreased membrane stability in conjunction with elliptocytic morphology has previously been documented in hereditary elliptocytosis due to primary skeletal protein defects that affect these two protein associations. Defective spectrin oligomerization as a result of a number of qualitative defects in α-spectrin, as well as quantitative protein 4.1 deficiency are the two common causes of decreased membrane stability. Our finding of the normal state of spectrin oligomerization and normal membrane content of protein 4.1 rule out the possibility that the decreased membrane stability of β and γ SGP-deficient cells could have resulted from primary skeletal protein defects. What is more important, these data allow us to identify yet another membrane protein association important in regulating membrane mechanical properties, the association of the cytoplasmic domain of a transmembrane protein with skeletal proteins. Because proteins analogous to erythrocyte protein 4.1 have been identified in various nonerythroid cells,[37-40] the present findings raise the possibility that the membrane physical properties of these cells may also be regulated by similar association between cytoplasmic domains of transmembrane proteins and components of the membrane skeleton.

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Studies on the Ultrastructure of Fibrin Lacking Fibrinopeptide B (β-Fibrin)

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Release of fibrinopeptide B from fibrinogen by copperhead venom procoagulant enzyme results in a form of fibrin (β-fibrin) with weaker self-aggregation characteristics than the normal product (αβ-fibrin) produced by release of fibrinopeptides A (FPA) and B (FPB) by thrombin. We investigated the ultrastructure of these two types of fibrin as well as that of β-fibrin prepared from fibrinogen Metz (Aα16 Arg→Cys), a homozygous dysfibrinogenemic mutant that does not release FPA. At 14°C and physiologic solvent conditions (0.15 mol/L of NaCl, 0.015 mol/L of Tris buffer pH 7.4), the turbidity (350 nm) of rapidly polymerizing αβ-fibrin (thrombin 1 to 2 U/mL) plateaued in <6 min and formed a "coarse" matrix consisting of anastomosing fiber bundles (mean diameter 92 nm). More slowly polymerizing αβ-fibrin (thrombin 0.01 and 0.001 U/mL) surpassed this turbidity after ≥60 minutes and concomitantly developed a network of thicker fiber bundles (mean diameters 118 and 186 nm, respectively). Such matrices also contained networks of highly branched, twisting, "fine" fibrils (fiber diameters 7 to 30 nm) that are usually characteristic of matrices formed at high ionic strength and pH. Slowly polymerizing β-fibrin, like slowly polymerizing αβ-fibrin, displayed considerable quantities of fine matrix in addition to an underlying thick cable network (mean fiber diameter 135 nm), whereas rapidly polymerizing β-fibrin monomer was comprised almost exclusively of wide, poorly anastomosed, striated cables (mean diameter 212 nm). Metz β-fibrin clots were more fragile than those of normal β-fibrin and were comprised almost entirely of a fine network. Metz fibrin could be induced, however, to form thick fiber bundles (mean diameter 76 nm) in the presence of albumin at a concentration (500 μmol/L) in the physiologic range and resembled a Metz plasma fibrin clot in that regard. The diminished capacity of Metz β-fibrin to form thick fiber bundles may be due to impaired use or occupancy of a polymerization site exposed by FPB release. Our results indicate that twisting fibrils are an inherent structural feature of all forms of assembling fibrin, and suggest that mature β-fibrin or αβ-fibrin clots develop from networks of thin fibrils that have the ability to coalesce to form thicker fiber bundles.

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THROMBIN-CATALYZED fibrin formation begins with the release of fibrinopeptide A (FPA) from the amino terminus of the Aα chains of fibrinogen[1-5] and is accompanied by the slower release of fibrinopeptide B (FPB) from Bβ chains. Reptilase, a snake venom enzyme from *Bothrops atrox*, removes only FPA,[6-8] release of which is sufficient for fibrin polymerization (α-fibrin) to occur.[7-9]

Mature fibrin clots evidently assemble from a network of thin protofibrils that subsequently, under physiologic solvent conditions, undergo lateral aggregation, causing increased fiber thickness.[10-12] Since 1947, two extreme forms of three-dimensional fibrin networks, namely "fine" and "coarse," have been recognized.[13] The fine gel structure can be produced at high ionic strength and pH, is translucent and friable, and consists of a network of thin, highly branched, twisting fibers.[14] The coarse gel structure, which forms at or below physiologic ionic strength and pH, is opaque and easily synerized. Owing to a strong tendency for lateral association under such conditions, these matrices are characterized by thick fiber bundles, the thicknesses of which are reflected by turbidity measurements[10-13,15-18] and determined in part by the kinetics of fiber growth.[11,12,18,19] That is, slowly forming clots at physiologic pH and ionic strength tend to develop thicker fiber bundles than do more rapidly forming clots.[12,18,20]

Some investigators[2,3,5,8,21] have suggested that FPB release is essential for lateral fiber growth. Other studies, however, indicate that this is not the case,[10,11,16,22,23] since reptilase cleavage of FPA to form α-fibrin results in a coarse matrix whose cables are only moderately narrower than those formed by αβ-fibrin.[10,11,22] The light scattering studies of Müller and co-workers[24] also indicated that α-fibrin and αβ-fibrin were similar with respect to branching tendency and lateral aggregation. Thus, although FPB release is not strictly required for gelation or for lateral aggregation, its release may increase the rate and extent of lateral associations.[10,11,16] In additon, fiber slippage is reduced, fiber stiffness is increased, and branch points in αβ-fibrin fibers are probably reinforced by noncovalent bonding correlating with FPB release.[14,23,25] Mihalyi and Donovan[26] recently provided calorimetric data indicating that noncovalent interactions of the fibrin(ogen) D domains are enhanced by removal of FPB.

Preferential release of fibrinopeptide B by copperhead venom procoagulant enzyme (CVE)[27] is sufficient to cause normal fibrinogen to polymerize.[28,29] Based on their gross appearance, these clots tend to be fine at temperatures

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<25°C and are readily dissociated at 37°C.[28,29] Judging from light scattering measurements on fibrin clots formed at 20°C and 37°C, it apears that selective cleavage of FPB at low enzyme concentrations results in the highest branching density and the lowest degree of lateral aggregation.[24]

In this study, we used electron microscopy to investigate the three-dimensional structure of β-fibrin formed from normal fibrinogen and from the homozygous congenital fibrinogen abnormality known as fibrinogen Metz (Aα Arg 16 → Cys).[30-32] To preserve the structures of these fragile fibrin clots, we prepared our material using the critical point drying technique[33] as recently used for the study of fine fibrin matrices.[14]

MATERIALS AND METHODS

Normal fibrinogen fraction I-2 (>95% coagulable) and fibrinogen Metz fraction I-2 were purified from citrated plasma by the method of Blombäck and Blombäck.[34] In some experiments, chromatographically purified normal peak 1 fibrinogen[35] was used. As assessed by the position of the fibrinogen band in $NaDodSO_4$-polyacrylamide gel electrophoresis (PAGE) (5% acrylamide),[36,37] unreduced fibrinogen Metz molecules were devoid of polymeric forms and the preparation was ~95% pure.

Sulfhydryl titrations of various fibrinogen preparations were carried out either with Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB),[38] or with ^{3}H- or ^{14}C-iodoacetic acid (specific activities 140 mCi/mmol and 11.1 mCi/mmol, respectively). After reaction with the iodoacetate, samples were extensively dialyzed, dissolved in 10 mL of Aquasol-2 liquid scintillation cocktail (New England Nuclear, Boston), and radioactivity was subsequently determined in a Packard model 2425 spectrometer.

Freeze-dried human thrombin (H-1), a gift from Dr D. L. Aronson, was reconstituted in H_2O and stored frozen at a concentration of 100 U/mL. CVE was prepared as described by Herzig and co-workers[27] and had an activity of 33 TAME (Nα-p-tosyl-L-arginine methyl ester) U/mL. Common chemicals were of reagent grade.

Fibrinogen samples for fibrinopeptide analyses were brought to a final concentration of 5 mg/mL in a buffer of 0.15 mol/L NaCl, 0.01 mol/L of Tris pH 7.2 ± 0.1, and incubated in small glass tubes after the addition of thrombin or CVE. Release of FPA and/or FPB from fibrinogen was measured after overnight incubation using the high-performance liquid chromatography (HPLC) chromatographic system described by Kehl and Henschen.[39] Specifically, the fibrinopeptides were analyzed with a Varian model 5040 HPLC apparatus on an Ultrasil ODS reverse-phase column (25 cm × 4.6 mm) (Rainin Instrument, Woburn, MA) using a linear gradient of acetonitrile (12% to 28%) buffered with 25 mmol/L of ammonium acetate pH 6.0.

αβ-Fibrin or β-fibrin was prepared by addition of thrombin or CVE (0.66 or 0.13 U/mL), respectively, at 14 ± 1°C, to normal fibrinogen, followed by overnight incubation at 14°C. Metz β-fibrin was generated at 14°C or 37°C by addition of CVE (0.66 U/mL) or thrombin (1 to 5 U/mL). In some cases, bovine serum albumin (BSA) (500 μmol/L, final concentration; Sigma Chemical, St Louis) or $CaCl_2$ (5 mmol/L), or both were included in the clotting mixture.

HPLC determination of fibrinopeptides released from normal β-fibrin under these conditions revealed the expected presence of FPB and des-Arg FPB and ≤10% of the potentially available amount of FPA. Metz β-fibrin showed only FPB and des-Arg FPB. For those batches of CVE that contained Aα chain degradative activity, tosyl lysine chloromethyl ketone (Sigma Chemical) 10^{-4} mol/L, final concentration, was added to the clotting mixture prior to CVE addition to block this activity. Turbidity measurements at 350 nm on fibrin clots were carried out at 14 ± 1°C in a Gilford model 240 spectrophotometer equipped with a jacketed specimen holder.

Certain β-fibrin specimens were subjected to gel-sieving chromatography at 37°C to separate β-fibrin from a minor αβ-fibrin contaminant.[29] In brief, following the incubation period with CVE, the clot was overlaid with 1 mmol/L of phenylmethylsufonylfluoride (PMSF) in buffer and incubated in the presence of this reagent for at least 2 hours. The fibrin was then dissociated by being warmed to 37°C, mixed with an equal volume of 0.3 mol/L of NaCl, 0.03 mol/L of Tris buffer pH 7.4 ± 0.1 PMSF 10^{-4} mol/L, and gel-filtered at 37°C through a Sepharose 2B column equilibrated in this same buffer. Early eluting fractions containing the αβ-fibrin contaminant were discarded, whereas fractions containing "β-fibrin monomer" eluting at a concentration above six-tenths of the β-fibrin peak height were pooled and stored frozen at −30°C at concentrations ranging from 400 to 800 μg/mL. For electron microscopy or turbidity measurements, aliquots of β-fibrin monomer were thawed at 37°C for 30 minutes, diluted 1:1 with H_2O, and then incubated at 14°C for the desired amount of time. Preparations were monitored by $NaDodSO_4$-PAGE[36,37] to verify whether anticipated fibrinopeptide cleavages had occurred and to determine if other proteolytic attack had occurred (eg, on Aα chains).

Samples of purified fibrin for negative staining electron microscopy were formed at 14 ± 1°C as 50-μL drops (50 to 500 μg/mL) in a Petri dish that had been humidified with a moist sponge, incubated for 16 to 18 hours with thrombin or CVE, picked up on glow-discharged carbon films, and then negatively contrasted with 2% (wt/vol) uranyl sulfate. For processing by the critical point drying technique, clots were picked up on glow-discharged carbon-coated 200-mesh copper grids, fixed with glutaraldehyde (2.5%, vol/vol) in 0.1 mol/L of HEPES buffer pH 7.0, containing 0.2% (wt/vol) tannic acid, washed several times with H_2O, stained with 0.5% (wt/vol) uranyl acetate, and washed again with H_2O.[14] The specimen was then dehydrated in graded ethanol solutions (20% to 100%, vol/vol) and CO_2 critical point dried[33] using a Tousimis model 780A critical point drying apparatus (Tousimis Scientific Instruments, Rockville, MD).

Plasma fibrin clots were prepared from blood that had been drawn into a plastic syringe, immediately transferred to a centrifuge tube, and then centrifuged at 3,000 g for 3 to 5 minutes to remove cellular elements. The supernatant plasma was incubated overnight at room temperature, and the resulting clot was synerized, washed extensively with saline, and fixed overnight at 4°C in 2% (wt/vol) glutaraldehyde solution in 0.1 mol/L of phosphate buffer pH 7.2. Metz fibrin clots were also prepared without syneresis from purified Metz fibrinogen at 2 mg/mL. The fixed preparation was incubated for 90 minutes with 2% osmium tetroxide in 0.1 mol/L of phosphate buffer, dehydrated in graded ethanol solutions, and embedded in Spurr's low-viscosity medium.[40] Thin sections were prepared using a diamond knife and a Reichert Ultracut ultramicrotome and stained with 5% aqueous uranyl acetate and Sato's lead citrate solution.[41]

Electron microscopy was performed with a Philips model 400 transmission electron microscope at 60 to 120 kV or with the University of Wisconsin high-voltage (1,000 kV) electron microscope at Madison, WI. Stereo images were made at magnifications of 5,000x to 22,000x at tilt angles of 3° to 6°.

RESULTS

We prepared β-fibrin at 14°C to minimize cleavage of FPA by CVE,[28] and we used this same temperature for polymerization of other forms of fibrin. The standard way of processing fibrin for transmission electron microscopy (TEM) is to pick up a clot sample by passing a carbon-coated grid through a fibrin clot. The grid surface is washed subsequently with buffer and uranyl sulfate and then air dried. Samples processed in this way have been suitable for observing the structure of a normal coarse fibrin matrix[42,43] and for characterizing the structure of certain abnormal fibrins such as fibrinogen Paris I.[44] The Metz clot structure and that of β-fibrin was so fragile, however, that at best we found discontinuous short strands and occasionally anastomosing fiber bundles and thin fibrils adherent to the underlying substrate. To preserve the three-dimensional structure of this type of clot, we processed the sample using the critical point drying procedure.[33] This technique has been used for demonstrating previously unrecognized features of fine clot matrices.

αβ-Fibrin. Images of critical point dried, rapidly polymerizing αβ-fibrin (thrombin, 1 U/mL) formed at 14°C under physiologic conditions (ie, I ~0.15, pH 7.4), revealed a coarse matrix consisting predominantly of twisted anastomosing cables (Fig 1A, Table 1). These cables showed typical cross-striations (~22 nm period) when viewed at a higher magnification than that shown in Fig 1 (data not shown).*

At a 100-fold lower thrombin concentration (0.01 U/mL), the rate of fibrin polymerization was considerably slowed (Fig 3); nevertheless, the clot ultimately developed considerably greater turbidity than fibrin formed at 1 U/mL. These observations confirm studies indicating that slow rates of fibrin polymerization favor higher degrees of lateral aggregation (ie, thicker fiber bundles).[12,18,20] At a thrombin concentration of 0.001 U/mL, clot development proceeded even less rapidly than at 0.01 U/mL although the turbidity eventually exceeded that of rapidly polymerizing αβ-fibrin. NaDodSO$_4$-PAGE analysis of fibrin specimens taken at the time of fixation for critical point drying, indicated that complete FPA release had occurred in all cases, but that in the specimen prepared at a thrombin concentration of 0.001/mL, only 50% of FPB had been released.

Consistent with turbidity measurements, fibrin formed at 0.01 U/mL or 0.001 U/mL revealed a network predominantly composed of large anastomosing fiber bundles that tended to be thicker than those from rapidly polymerized αβ-fibrin (Fig 1). In addition to the thick cable network, however, there was also a prominent network of thinner fibrils. This second type of matrix was most prominent at the lowest thrombin concentration (0.001 U/mL) and consisted of highly branched, twisting fibrils (Fig 4) that appeared to entrap and merge with large fiber bundles. The thick cables forming under this latter condition were composed of loosely packed fibrils (arrows, Fig 1C) and did not display the striations typical of more tightly packed bundles.

β-Fibrin. β-Fibrin gels formed at 14°C can be dissociated by being warmed to 37°C, and the resulting β-fibrin monomer can subsequently be polymerized by incubation at room temperature or below.[28,29] This process can be carried out repeatedly on material that has been inactivated with PMSF. Turbidity measurements on such a β-fibrin monomer preparation (Fig. 5) indicated that the onset of polymeriza-

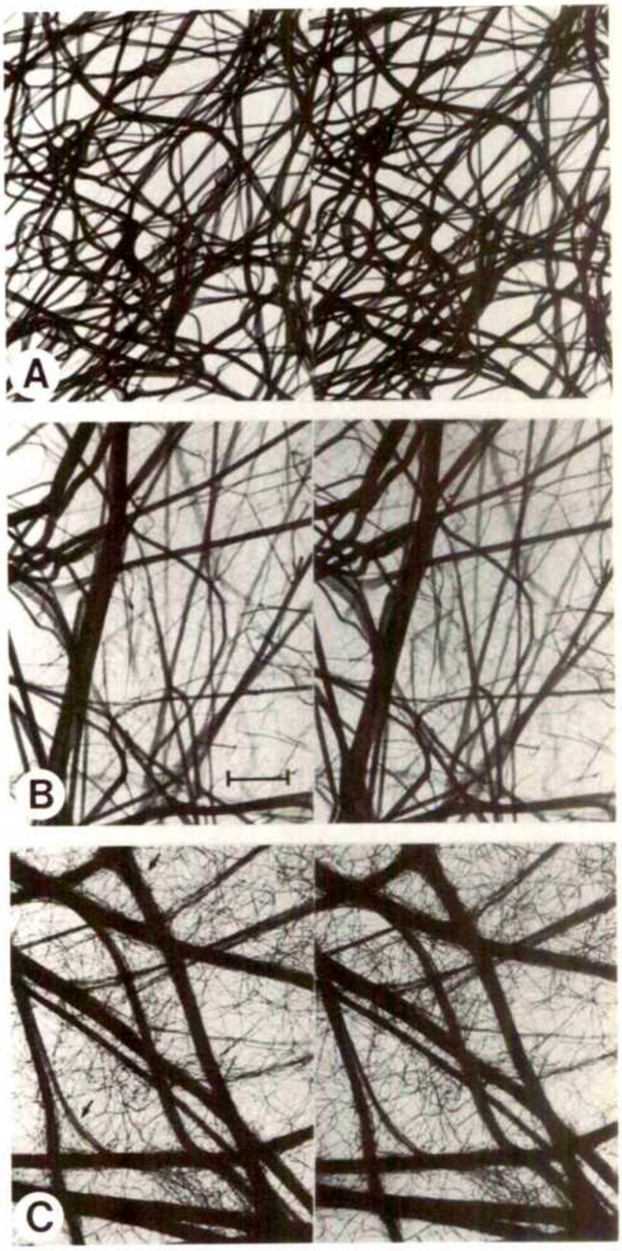

Fig 1. Stereo images by transmission electron microscopy (TEM) of αβ-fibrin (500 μg/mL) prepared by incubation overnight at 14°C under "coarse clot" buffer conditions (0.15 mol/L of NaCl, 0.015 mol/L of Tris buffer, pH 7.4) at the following thrombin concentrations: (A) 1 U/mL; (B) 0.01 U/mL; (C) 0.001 U/mL. Bar = 1,000 nm. The arrows in (C) indicate loosely packed fiber bundles.

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*Beam penetration of the thick fiber bundles that form in rapidly polymerizing α,β-fibrin or other densely packed fibers at relatively low magnification (eg, 10,000×) is poor using TEM even at 120 kV. Striations are readily observed in thin sections of such fibers, (eg, Fig 2A), in critical point dried specimens at higher magnification (eg, 20,000×), or using higher voltages (eg, high-voltage electron microscopy, HVEM).

Table 1. Fiber Diameter of Thick Fiber Bundles of $\alpha\beta$-Fibrin and β-Fibrin

Preparation	Concentration (μg/mL)	n*	Fiber Diameter (nm) Mean	SD	Range
$\alpha\beta$-Fibrin (thrombin, 2 U/mL)	50	36	83	±37	50–170
$\alpha\beta$-Fibrin (thrombin, 1 U/mL)	500†	89	92	±39	36–260
$\alpha\beta$-Fibrin (thrombin, 0.01 U/mL)	500	43	118	±59	35–256
$\alpha\beta$-Fibrin (thrombin, 0.001 U/mL)	500	36	186	±90	60–536
β-Fibrin monomer	355	55	212	±80	40–520
β-Fibrin (CVE, 0.66 U/mL)	500	36	87	±30	35–170
β-Fibrin (CVE, 0.13 U/mL)	500	48	135	±73	35–421
Metz β-fibrin + albumin + Ca^{2+}	500	29	76	±32	39–194

In matrices containing significant amounts of both "fine" and "coarse" networks, there appeared to be no sharp size transitions in the progression from thin fibers in the fine matrix (7 to 30 nm in diameter) toward thicker fibers approaching the mean values. For purposes of this analysis, we defined the diameter of a coarse fiber bundle as ≥35 nm.

*Number of fibers measured.

†Owing to the thickness of the clot in the critical point dried specimen, it seemed tenuous to assume that areas selected for fiber measurement were representative of the fiber population in the clot. For that reason, we also prepared thin sections of an imbedded specimen under these same conditions. The fiber diameter determined from this type of specimen preparation was 92 ± 26 nm, range 37–150 nm. SD and range of values were lower because we used only the minimum cross-sectional diameter of any given fiber to avoid including measurement of tangential or oblique sections of fibers in our calculations.

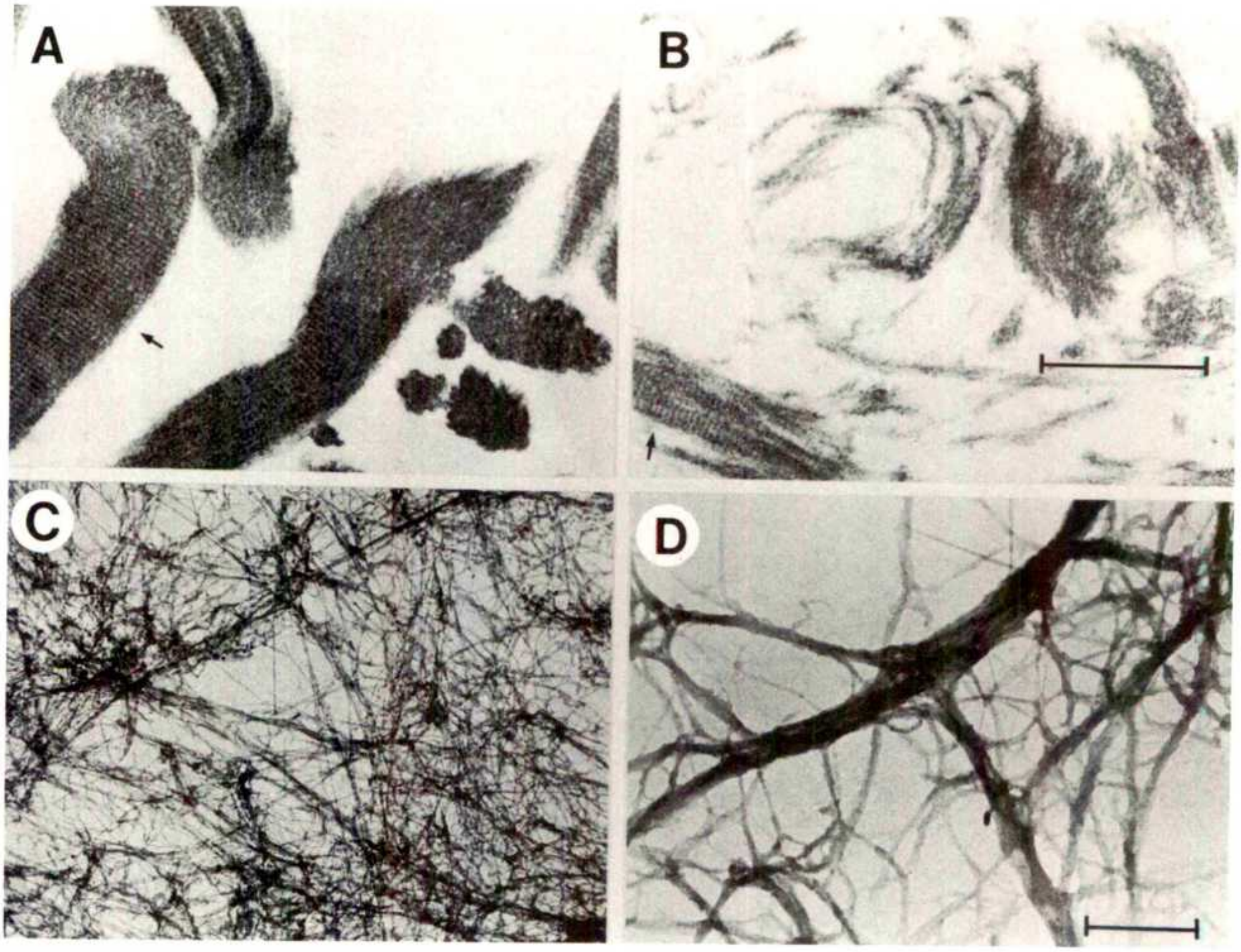

Fig 2. Comparison of the ultrastructure of a Metz plasma fibrin clot with Metz fibrin formed in the presence of albumin or Ca^{2+} or both (TEM). (A) Thin section of a normal plasma fibrin clot; (B) thin section of a Metz plasma clot; bar = 500 nm (A and B); (C) critical point dried specimen of Metz fibrin prepared in the presence of 5 mmol/L of $CaCl_2$; (D) critical point dried specimen of Metz fibrin prepared with albumin 500 μmol/L and $CaCl_2$ 5 mmol/L; bar = 500 nm (A and B). Arrows (A and B) point to periodic striations that occur in thick fiber bundles.

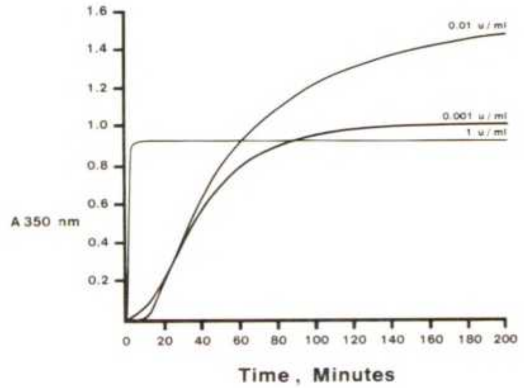

Fig 3. Turbidity measurements on $\alpha\beta$-fibrin. These experiments were carried out under the same conditions described in Fig 1. Thrombin was added to a final concentration of 1 U/mL, 0.01 U/mL, or 0.001 U/mL; absorbance (A_{350} nm) was monitored as a function of time.

tion was more rapid than that of β-fibrin formed by direct addition of CVE (Fig 6). The initial rate of turbidity rise of β-fibrin forming at the higher CVE concentration (0.66 U/mL), however, was about the same as the value for β-fibrin monomer extrapolated to a concentration of 500 μg/mL (ie, 0.061/min *v* 0.065/min). Electron micrographs of the β-fibrin monomer clot showed a network of twisting, occasionally anastomosing cables (Fig 7A). The mean fiber diameter was greater than in any other type of β-fibrin or αβ-fibrin matrix (Table 1).

β-Fibrin produced by addition of CVE (Fig 7) formed a network of thick cables frequently displaying periodic striations (see footnote, page 1075) typical of α-fibrin or αβ-fibrin bundles. The fiber bundles in these networks, like the β-fibrin monomer network, anastomosed only occasionally relative to αβ-fibrin clots. At the highest CVE level (0.66 U/mL), the matrix revealed occasional areas of fine, fibrillar networks, usually bridging between thick cables (Fig 7B) or appearing to represent an unraveled or frayed portion of a thick fiber bundle. At a lower CVE level (0.13 U/mL), fiber bundles in the matrix were more loosely packed (Fig 7C, arrow) and wider than those formed at the higher CVE concentration. In addition to the predominant thick cable structure, this clot showed a considerable network of fine twisting fibrils (Fig 7C and Fig 4B).

Metz β-fibrin. Metz β-fibrin is more transparent, less viscous, and more easily disrupted than normal β-fibrin. In contrast to normal, Metz β-fibrin formed by direct addition of thrombin or CVE at 14°C or that formed at 14°C from Metz β-fibrin monomer solutions (ie, first incubated with thrombin at 37°C), revealed a matrix comprised almost entirely of thin, twisting, and highly branched fibrils (Figs 4C and 7D) that occasionally coalesced to form somewhat thicker, relatively short, uneven bundles. These observations are consistent with the very low turbidity recorded at 350 nm (Fig 6).

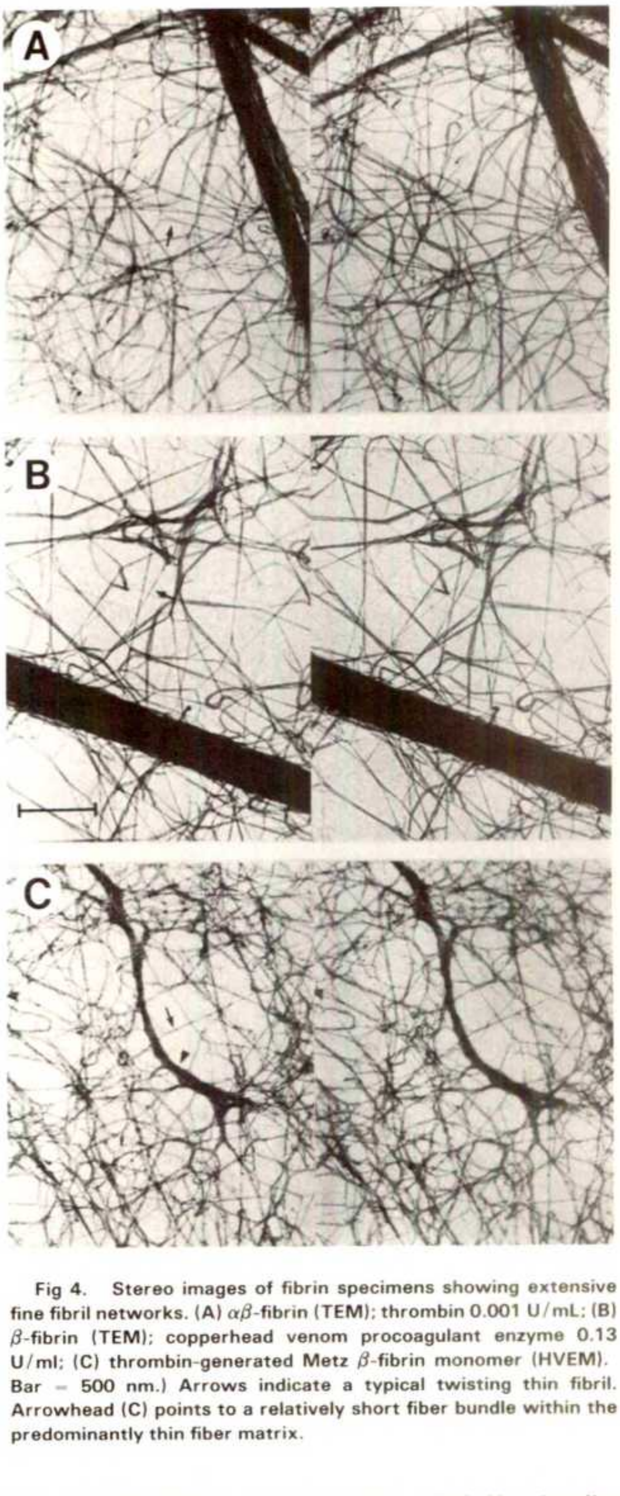

Fig 4. Stereo images of fibrin specimens showing extensive fine fibril networks. (A) αβ-fibrin (TEM); thrombin 0.001 U/mL; (B) β-fibrin (TEM); copperhead venom procoagulant enzyme 0.13 U/ml; (C) thrombin-generated Metz β-fibrin monomer (HVEM). Bar = 500 nm.) Arrows indicate a typical twisting thin fibril. Arrowhead (C) points to a relatively short fiber bundle within the predominantly thin fiber matrix.

To evaluate the type of Metz fibrin matrix formed in vivo, we prepared fibrin directly from native plasma and examined thin sections by electron microscopy (Fig 2). Normal fibrin yielded the expected thick, striated cables (Fig 2A, arrow), whereas Metz fibrin yielded loosely packed fiber bundles that frequently were sufficiently in register to yield a striated pattern (Fig 2B, arrow). Fibrin aggregation is well known to accelerate in plasma,[45] and this is due in part to effects related to the presence of albumin and IgG.[46,47] The accelerating effect is also accompanied by increased clot turbidity that is attributable, at least in part, to the presence of albumin or IgG, or both,[47] and Ca^{2+}.[48,49] To provide an explanation for the more mature structure of Metz plasma

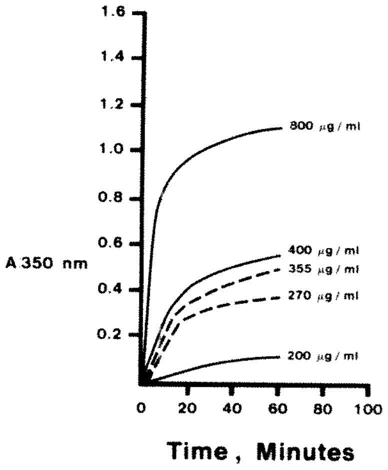

Fig 5. Turbidity measurements on β-fibrin monomer. Repolymerization at 14°C under standard buffer conditions of β-fibrin monomer that had been dissociated at 37°C (—) or further processed after dissociation at 37°C by molecular exclusion chromatography to remove $\alpha\beta$-fibrin contamination (---). The curves developed by chromatographically processed β-fibrin monomer are in register with those of unprocessed β-fibrin monomer, suggesting that a small amount of $\alpha\beta$-fibrin contamination does not materially change the structure of the matrix that forms.

fibrin relative to purified thrombin-generated or CVE-generated Metz fibrin, we attempted to induce thick fibril formation by allowing thrombin-generated Metz fibrin to polymerize in the presence of albumin (500 μmol/L) or 5 mmol/L of $CaCl_2$, or both (Fig 6). Measurements in the presence of albumin showed increased turbidity developing slowly over several hours; this effect was even more marked when 5 mmol/L of $CaCl_2$ was also present, although addition of $CaCl_2$ alone to Metz fibrin had little effect (Figs 6 and 2C). Examination and measurement of critical point dried fibrin specimens confirmed that increased thick bundle formation had occurred in albumin-containing specimens (cf Fig 2C and 2D). The thick bundles, like the fine fibrils, had a tendency to twist, but periodic striations were not observed. As assessed by TEM of thin sections, thick fiber bundle formation could also be induced in Metz β-fibrin formed at a concentration approximating that in plasma (ie, 2 mg/mL) (data not shown).

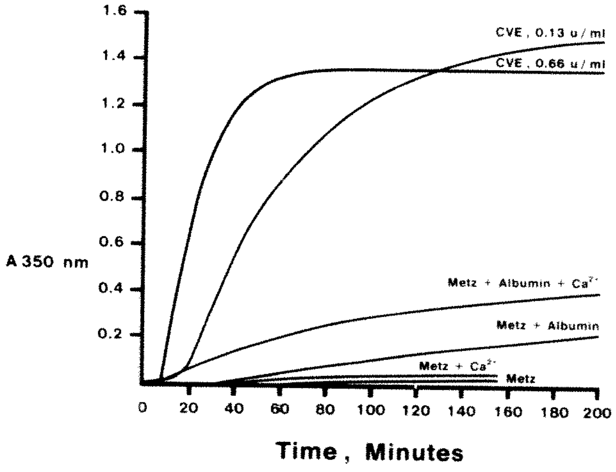

Fig 6. Turbidity measurements on β-fibrin (500 μg/mL). CVE additions, 0.66 or 0.13 U/mL, to normal fibrinogen at 14°C were made under the same buffer conditions described in Fig 1. Metz β-fibrin was incubated with thrombin (5 U/mL) at 14°C and allowed to polymerize under the following conditions: Metz + buffer; Metz + $CaCl_2$, 5 mmol/L; Metz + albumin, 500 μmol/L; Metz + albumin, 500 μmol/L + $CaCl_2$, 5 mmol/L.

Sulfhydryl content. Gel electrophoretic analysis of fibrinogen Metz, as well as electron micrographs of negatively contrasted Metz fibrinogen (not shown), revealed that Metz molecules were monomolecular and thus were not joined by intermolecular covalent (ie, disulfide) bridges. To investigate further whether the cysteine residues in fibrinogen Metz at position 16 were oxidized to disulfides, we evaluated the titratable sulfhydryl (SH) content using Ellman's reagent.[38] Normal fibrinogen, which has no free SH groups,[50] and Metz fibrinogen yielded <0.2 mol of titratable SH groups per mole of fibrinogen. We also used a more sensitive technique to detect the presence of SH groups by labeling with ^3H or ^{14}C iodoacetic acid. With this technique, the titratable SH content of Metz fibrinogen as well as that of normal peak 1 fibrinogen was 0.01 to 0.02 mol of SH/mol of fibrinogen.

DISCUSSION

Although many EM studies have been made of the structure of α-fibrin and $\alpha\beta$-fibrin, to our knowledge none have reported on the ultrastructure of β-fibrin. By using the critical point drying technique to process fibrin clots, we were able to preserve clot structure and obtain high-quality, detailed stereo images of β-fibrin and Metz β-fibrin and Metz β-fibrin clot networks. Our findings, coupled with turbidometric analyses, permitted a comparison of the structure of $\alpha\beta$-fibrin with that of β-fibrin and Metz β-fibrin.

It is clear from our present findings that release of FPB alone can give rise to clots whose features resemble those of α-fibrin in several important ways: (a) Both types of fibrin tend to form thick twisting cables displaying typical periodic cross-striations under physiologic buffer conditions. When polymerization takes place relatively slowly, both types of fibrin develop fine fibrillar network structures in addition to a relatively thicker cable network. The latter observation is consistent with previous studies indicating that fiber thickness is determined in part by the kinetics of fiber growth.[11,12,18-20] (b) The fibrils from both types of fibrin show the characteristic twisting described by Müller and colleagues[14] for fine clots. Regions containing fine fibrillar matrix structures, whether derived from normal (β-fibrin or $\alpha\beta$-fibrin) or Metz β-fibrin, are not easily distinguishable from the fine matrix formed by $\alpha\beta$-fibrin at high ionic strength/pH[14] or in a slowly polymerizing system under physiologic conditions (this study).

Twisting is not only a characteristic of native fibrin fibrils (ref. 14 and this study), but is also a prominent feature of microcrystals and fibrin clots prepared from enzymatically modified fibrinogen.[51,52] We infer from those results and from our present observations that fiber twisting is an inherent feature of fibrin assembly when polymerization is initiated by release of either FPA or FPB.

Slowly polymerizing αβ-fibrin or β-fibrin networks contain fine fibrillar substructures that are superimposed on and merge with thick, loosely packed fiber bundle networks. Such structures evidently reflect an intermediate phase of fibrin assembly during which thick cables coalesce from a fine fibrillar matrix to form the mature anastomosing cable network. This observation is consistent with the observations that under physiologic conditions mature fibrin assembly develops over a relatively long time[23] from protofibrils that subsequently undergo lateral aggregation to form thick fiber bundles.[10-12] The precise mechanism by which this assembly process takes place is not yet known.

Clot turbidity is reportedly a function of the second power of fiber diameter.[15,17,53] Therefore, such measurements are sensitive to the presence of thick fiber bundles but not to fine fiber networks. The widest cable structures we observed occurred in the coarse β-fibrin monomer clots, yet these gels ultimately developed lower turbidity than did the rapidly polymerizing coarse αβ-fibrin matrix or any other β-fibrin or αβ-fibrin network having lower mean fiber widths (Table 1). This seemingly paradoxical phenomenon may indicate that light scattering per se is not strictly a function of fiber width but may also be related to factors such as tightness of fiber packing, branching, etc. Further studies combining electron microscopy with detailed physical and rheological measurements of clot properties should be most useful in clarifying this issue.

In contrast to turbidity, measurement of clot permeability is a reflection of pore size, coarse clots having greater permeability than fine clots.[15,17-20] Shah and colleagues[17,18] observed that the permeability of fibrin formed at a low thrombin concentration did not rise in proportion to the increase in fiber thickness calculated from turbidity measurements. Such observations are consistent with our present demonstration that slow polymerization (αβ-fibrin or β-fibrin) is accompanied not only by increased fiber thickness, but also by the formation of fine fiber networks. Deductions on clot matrix structure based on turbidity and permeability measurements depend on the assumption of a uniform population of network fibers. This is evidently not the case (ref. 17 and this study), especially under circumstances that are intermediate to the extremes of fine and coarse fiber network formation.

Release of FPA and FPB exposes independent binding sites in the NH_2-terminal region of the molecule which appear to function cooperatively in the aggregation process.[28,29] Release of FPA exposes the "A" site, which becomes aligned with the complementary "a" site in the D domain of another molecule.[5,21] Similarly, FPB release evidently exposes a "B" polymerization site,[28,29] efficient utilization of which by the complementary "b" site may depend on proper alignment of two D domains in an αβ-fibrin polymer.[21] Substitution of cysteine for arginine at Aα 16 in fibrinogen Metz evidently impairs this process because Metz β-fibrin, unlike normal β-fibrin, has only a weak tendency for lateral aggregation. This impairment may be due to the existence of a disulfide bridge at Aα 16.[54] The substitution may impose conformational or steric changes that interfere with proper occupancy or orientation of the polymerization site unmasked by release of FPB, thus impairing optimal alignment of fibrils into bundles. Further study of this impairment may lead to new insights into the exact location of the region involved in the ability of fibrin to form lateral associations with other fibrin molecules.

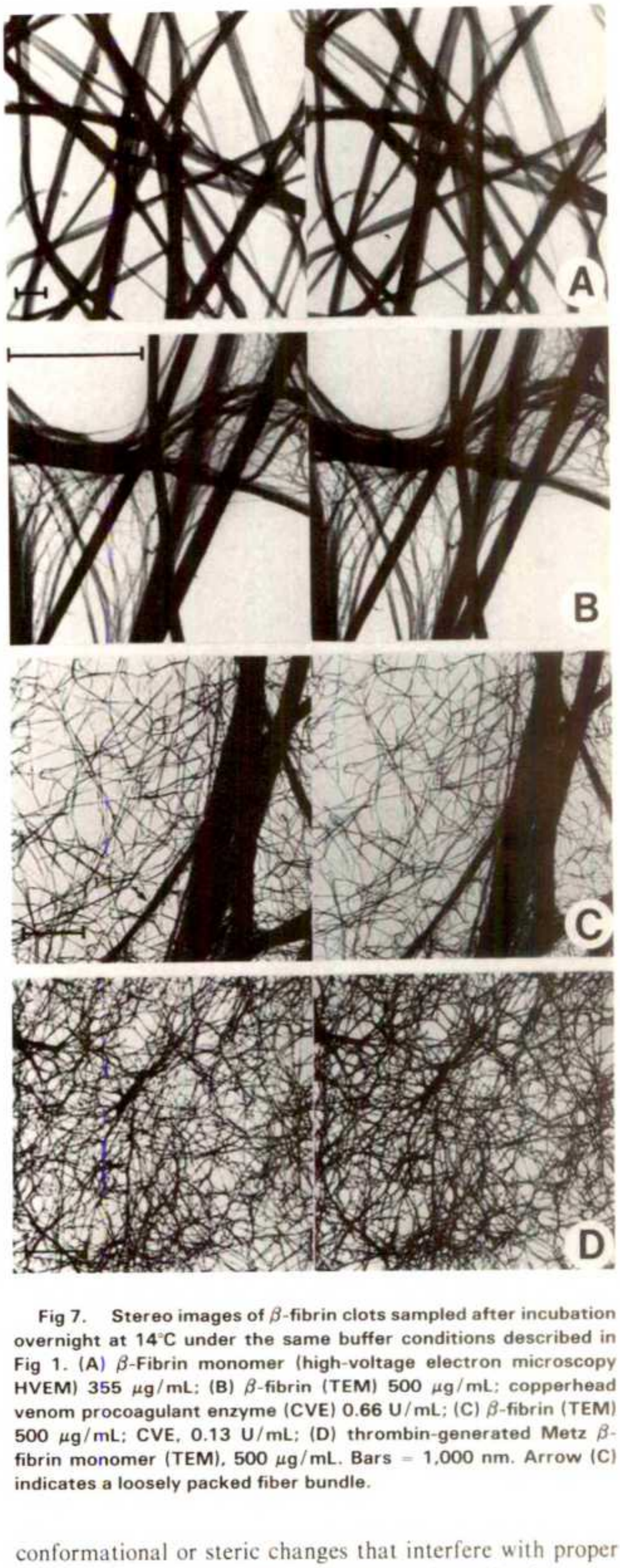

Fig 7. Stereo images of β-fibrin clots sampled after incubation overnight at 14°C under the same buffer conditions described in Fig 1. (A) β-Fibrin monomer (high-voltage electron microscopy HVEM) 355 μg/mL; (B) β-fibrin (TEM) 500 μg/mL; copperhead venom procoagulant enzyme (CVE) 0.66 U/mL; (C) β-fibrin (TEM) 500 μg/mL; CVE, 0.13 U/mL; (D) thrombin-generated Metz β-fibrin monomer (TEM), 500 μg/mL. Bars = 1,000 nm. Arrow (C) indicates a loosely packed fiber bundle.

In contrast to purified Metz fibrin, the Metz plasma clot was less fragile, could be synerized, and showed a considerable degree of lateral aggregation, even to the extent of forming easily discernible periodic striations (Fig 7). This "improvement" in Metz clot structure is not surprising since fibrin aggregation in plasma is associated with increased clot turbidity and increased lateral fiber aggregation.[47] When Metz fibrin was formed at concentrations approaching that in plasma or at an albumin concentration in the physiologic range (500 μmol/L), we were able to induce an increased degree of lateral fiber association. This effect was even more pronounced when Ca^{2+} was also present. We believe that the differences between purified Metz β-fibrin and plasma Metz β-fibrin structure are largely attributable to the fact that the plasma environment favors lateral fiber growth, due in part to the relative concentration of fibrinogen and to albumin or IgG.[46,47] Other plasma factors contributing to the strength and structure of the Metz plasma clot (eg, crosslinking) remain to be explored. In this regard, the Metz subject is not a severe or spontaneous bleeder, although she often bleeds for prolonged periods from venipuncture sites and is a habitual aborter.[30] Perhaps the relatively mild clinical bleeding that she manifests reflects the effect that the plasma environment has in enhancing fibrin clot formation.

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Early T Cell Differentiated Chronic Myeloid Leukemia Blast Crisis With Rearrangement of the Breakpoint Cluster Region but Not of the T Cell Receptor β Chain Genes

By M. Gramatzki, C.R. Bartram, D. Müller, M. Walter, H. Tittelbach, and J.R. Kalden

Early T cell differentiation is described in a case of Philadelphia chromosome-positive chronic myeloid leukemia (CML) in blast crisis, supporting multi-lineage differentiation potential of CML precursor cells. In the absence of myeloid markers, strong positivity for terminal deoxynucleotidyl transferase (TdT) and reactivity with T cell antibody 3A1, but lack of more mature T cell antigens, provided evidence for immature T cell differentiation. Molecular analysis of the breakpoint cluster region (*bcr*) in chromosome 22 revealed a rearrangement and thus confirmed the CML origin of the early T cell blasts. T cell receptor β chain sequences were found in germline configuration and therefore suggest a very immature stage of T cell differentiation in the CML blasts.

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CHRONIC MYELOID leukemia (CML) is believed to originate from a malignant transformation of a hematopoetic precursor cell, differentiated closely to the stem cell.[1] Although the common differentiation pathway of CML cells is myeloid, the occurrence of early lymphoid cells in certain cases of CML blast crisis,[2-4] isoenzyme studies,[5] the establishment of Philadelphia chromosome-positive mature B cell lines from CML patients,[6,7] and maturation studies of CML cell lines[8] suggest a multi-lineage differentiation potential of the malignant cell. Although approximately one-third of CML in blast crisis are lymphoid differentiated, T cell involvement has been distinctively rare and quite often disputed. Particularly in cases identified initially in blast crisis it may be difficult to assign the proper classification and to distinguish those from the Philadelphia chromosome-positive acute lymphoblastic leukemias (ALL).

More sophisticated cell surface marker analysis and gene rearrangement studies have recently become available. The use of such techniques in addition to characterization with conventional methods was applied to cells from a patient with CML blast crisis, and the diagnosis of early T cell CML could be established. Cytogenetical studies confirmed the diagnosis of CML by typical Philadelphia translocation[9] with rearrangement of the breakpoint cluster region (*bcr*).

Studies of the T cell receptor (TCR) β gene[10] revealed that these early T cell blasts had not yet been rearranged for the TCR. This report, for the first time, describes immunocytologically and genetically confirmed CML of early T cell differentiation lacking TCR β chain gene rearrangement.

MATERIAL AND METHODS

Immunocytological analysis. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Metrizoate density-gradient centrifugation, and staining procedures were used as previously described.[11] Monoclonal antibodies used included OKT3, OKT4, and OKI-1[12,13] (Ortho Pharmaceuticals, Raritan, NJ); Leu-2[14] and Leu-M1[15] (Becton Dickinson, Mountainview, CA); B1 and B4[13,16] and My-7[13] (Coulter, Hialeah, FL); BA-1 and BA-2[17] (Hybritech, San Diego); 24.1[18] kindly provided by Dr Hansen, Seattle; Mo-S39 and Mo-U28[19] a gift of Dr Burmester (Erlangen) and Dr Winchester (New York) and the anti-T cell reagent 3A1.[20] Antibodies TH69 and TC25 were raised in our laboratory.[21] Specificities are described in the Results section. The percentage of positive cells as well as antigen density were assayed in an indirect immunofluorescent test and evaluated by fluorescence microscopy as well as fluorescent-activated cell sorter (FACS) analysis (EPICS V, Coulter) as previously described.[11,22] Irrelevant antibodies were used as negative controls, and an anti-HLA-class I antibody was used as positive control when appropriate. Cytocentrifuge-prepared cells were evaluated for presence of terminal deoxynucleotidyl transferase (TdT) according to a method of Bollum.[23]

Chromosomal analysis. Slides were prepared from 24-hour cultures made from a frozen stock of PBMCs. Conventional chromosomal analysis was performed using quinacrine fluorescence banding techniques for identification of chromosomes, and classification was made according to the Paris nomenclature.[24]

DNA analysis. DNAs were extracted from mononuclear cells after Ficoll-Hypopaque centrifugation by standard techniques. Fifteen micrograms of DNA was digested with appropriate restriction enzymes (Boehringer, Mannheim, FRG), electrophoresed on a 0.7% agarose gel, blotted and hybridized to 3′ and 5′ *bcr* sequences,[25] a

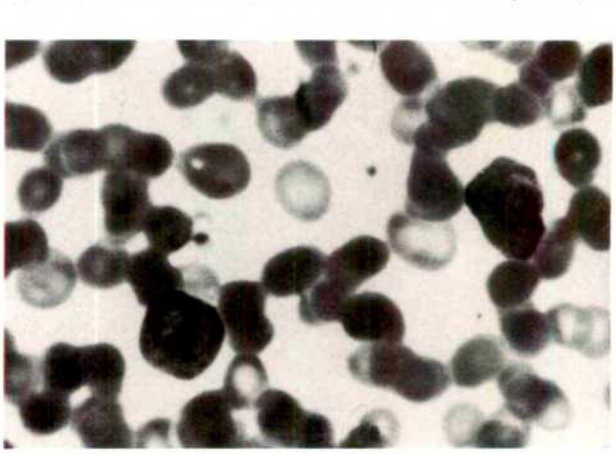

Fig 1. Leukemic blasts in the peripheral blood of patient E.S.

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Table 1. Phenotypic Analysis of the Patient's Blood Mononuclear Cells Using Monoclonal Antibodies

Antibody*	Predominant Specificity	Percentage of Positive Cells
OKT 3 (CD3)	Mature T cells	3
OKT 4 (CD4)	Helper T cell subset	0
Leu-2 (CD8)	Suppressor/cytotoxic T cell subset	0
3A1 (CD7)	Immature T cells and most mature T cells	60
TH 69	Immature T cells and subset of mature T cells	54
B 4 (CD19)	B cells, pre-B cells	0
B 1 (CD20)	B lymphocytes	0
BA-1 (CD24)	B lymphocytes, immature B cells	10
BA-2 (CD9)	Immature B cells, lymphoid precursors	1
24.1 (CD10)	Common-ALL antigen	0
Mo-S39 (CDw14)	Monocytes	1
Leu-M1 (CD15)	Myeloid precursors, monocytes, granulocytes	14
Mo-U28 (CDw13)	AML cells	10
My-7 (CDw13)	AML cells	8
TC 25	Immature T cell subset and CML blast crisis cells	50
OKI-1	Ia (HLA-DR) bearing cells	1

ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CML, chronic myeloid leukemia.

*CD classification in parentheses.

TCR β chain probe[26] and an Ig Cμ probe (kindly provided by Dr Leder). After hybridization, the filters were washed under stringent conditions and exposed to XAR-5 film (Kodak) using Dupont Lightning-Plus intensifying screens for 14 hours at −70°C.

Case report. A 53-year-old woman had a 6-month history of mild abdominal pain, weight loss, and low-grade fever. When she was admitted to the hospital, she had massive hepatosplenomegaly and, when examined hematologically had a WBC count of 113,000/μL. Anemia was mild, and her hemoglobin level was 9.8 g/dL. Thrombocytes were slighly elevated to 350,000/μL. Differential count revealed 79% blasts, 16% granulocytes, and 5% lymphocytes. The bone marrow aspirate demonstrated 90% blast cells of medium to large size with irregular, lobulated nuclear shape.

RESULTS

The patient's blast cells could not be classified into lymphoid or myeloid lineage by morphology alone (Fig 1). Cytochemical analysis of the blast cells revealed no reactivity in esterase, peroxidase, and periodic-acid Schiff (PAS) stains. TdT positivity, however, could be demonstrated in the indirect immunofluorescent test. Results of immunological analysis using a battery of monoclonal antibodies are shown in Table 1. Strong reactivity occurred with 3A1 (Fig 2), an antibody reacting with most mature and all immature T cells.[20] Blast cells reacted also with another anti-T cell antibody, designated TH69, which has specificity similar to that of 3A1 (ref. 21 and Gramatzki, unpublished observations). Early T cell differentiation was further suggested by absence of staining with the more mature anti-T cell antibodies[12,14] such as OKT3, OKT4, or Leu-2. Most cells expressed an antigen, detected by TC25, that is expressed only on some early T cell ALL, myelomonocytic and monocytic leukemias, and CML in blast crisis.[21] In contrast, <15% of the PBMCs of the patient had myeloid or monocytic antigens on their surface, as determined by antibodies Mo-S39, Leu-M1, Mo-U28, and My-7. Likewise, no evidence for B cell or immature non-T cell lymphoid differentiation was found using reagents B1, B4, BA-1, BA-2, and the anti-common ALL (CALLA) antibody 24.1. Ia-antigens were not detected, a familiar finding on malignant cells differentiated on the early T cell level.

Cytogenetic analysis revealed the presence of a Philadelphia chromosome (Fig 3). To study the Philadelphia translocation molecularly, configuration of the *bcr* gene residing on

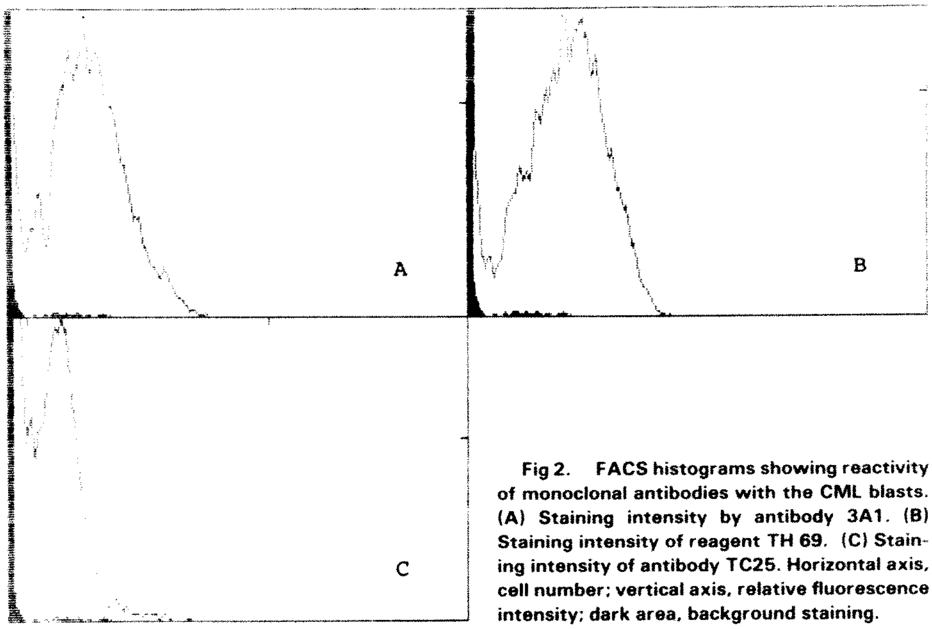

Fig 2. FACS histograms showing reactivity of monoclonal antibodies with the CML blasts. (A) Staining intensity by antibody 3A1. (B) Staining intensity of reagent TH 69. (C) Staining intensity of antibody TC25. Horizontal axis, cell number; vertical axis, relative fluorescence intensity; dark area, background staining.

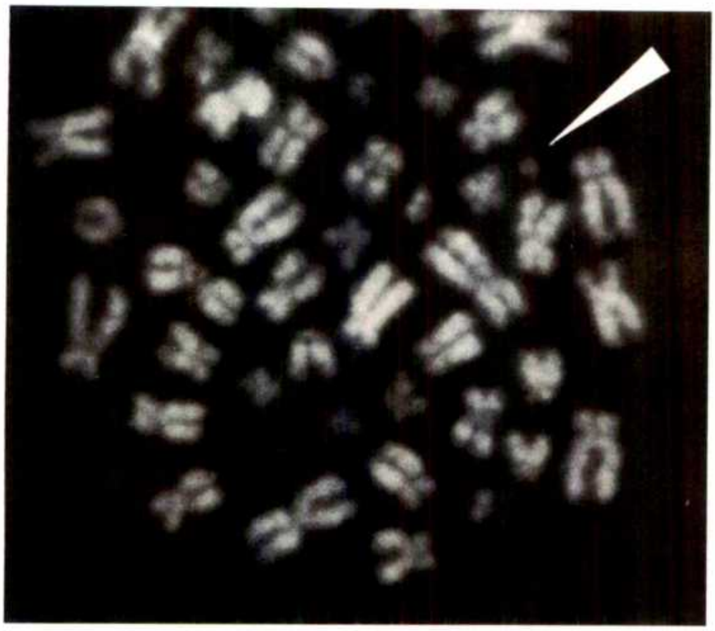

Fig 3. Q-banded metaphase exhibiting characteristic Philadelphia chromosome (arrow).

chromosome 22 was analyzed. Southern blot analysis revealed a rearrangement within *bcr* (Fig 4).

Because the blasts were typed as early T cell origin, whether the gene for the T cell receptor β chain was already rearranged was of interest. Southern blot analysis, however, showed germline configuration (Fig 5).

To exclude any pre-B cell differentiation on the molecular level, Cμ sequences were used to investigate immunoglobulin heavy chain genes. Not surprisingly, they were found in germline configuration (Fig 6).

DISCUSSION

Because T cell differentiation of blasts in CML is a rare event,[7,13] it appears important to review the evidence for classification in this case. Absence of esterase and peroxidase staining, and lack of reactivity with several antibodies detecting different myeloid antigens, but presence of TdT, an enzyme marker associated with lymphoid differentiation,[4,27] was highly suggestive of a lymphoid affiliation of the patient's leukemic cells. Reactivity with T cell antibody 3A1, dissecting immature T cells from pre-B cell forms,[17,20,22] and lack of staining with antibodies defining Ia antigens, B cells, or mature T cells, finally established differentiation on the early T cell level. This was confirmed by demonstration of immunoglobulin genes in germline configuration.

Although the clinical situation with massive spleen enlargement and thrombocytosis was already suggestive of CML in blast crisis,[28,29] Philadelphia chromosome positive ALL,[24,30-32] although almost always of non-T non-B cell type,[24] had to be excluded. Recently, molecular techniques have become available which appear to be helpful in this situation.[9,28,32] The typical Philadelphia translocation seen in CML patients places the *c-abl* oncogene adjacent to *5′bcr* sequences on chromosome 22q −, whereas *3′bcr* sequences are translocated to chromosome 9q +.[33] Because true Ph^1-

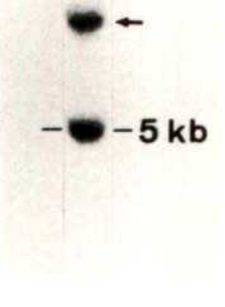

Fig 4. Southern blot analysis of *bcr* sequences; 15 μg of DNA obtained from peripheral blood cells and digested with BglII. Hybridization to a 5′ *bcr* probe detects a rearranged fragment (arrow) beside a 5-kilobase (kb) germline band.

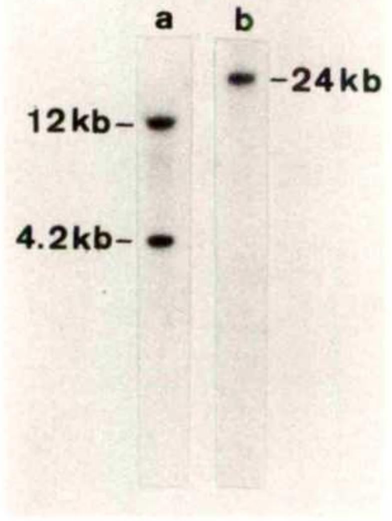

Fig 5. Configuration of T cell receptor β chain sequences. DNA samples (15 μg) were digested with *EcoRI* (a) and *BamHI* (b) restriction enzymes, electrophoresed, and hybridized to the $C_T\beta$ probe detecting 12-kilobase (kb) $C_T\beta_1$ and 4.2-kb $C_T\beta_2$ germline fragments in *EcoRI* and a 24-kb $C_T\beta$ germline fragment in *BamHI* digests.

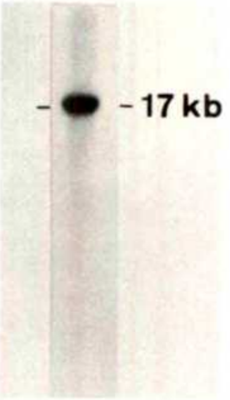

Fig 6. Southern blot analysis of $C\mu$ Ig sequences: 15 μg of DNA was digested with *Bam*HI and hybridized to a $C\mu$ probe that detects a 17-kilobase (kb) germline band.

positive ALL studied so far appeared to be different on the molecular level,[9,28,33-35] the typical *c-abl/bcr* rearrangement seen in our patient firmly establishes this T cell malignancy as a blast crisis of CML.

Recently, additional insight in T cell malignancies was obtained by studying the gene for the TCR,[10,36] and most but not all T cell tumors had the receptor genes clonally rearranged.[36-38] In this case of T cell CML, however, the TCR β gene was in germline configuration, which is in agreement with the immature differentiation level, since the receptor becomes rearranged during T cell maturation.[10] Very recently, two cases of T cell CML with already rearranged TCR were reported, in one of which typical *c-abl/bcr* rearrangement was documented as well.[39,40] The present study is the first report of a T cell CML blast crisis with TCR still in germline configuration, indicating heterogeneity of differentiation stages in T cell CML. This is supported by the few published cases of immunologically characterized T cell CML in which some were immature as well[41,42] and one apparently was late thymic-differentiated,[43] while Griffin and co-workers[13] reported a T cell CML with a mature phenotype that coexpressed myeloid antigens.

Our data provide definite evidence for the multilineage differentiation capacity for the CML precursor cell including the T cell lineage. Although T cell CMLs—despite their rarity—phenotypically are heterogeneous, they have certain molecular genetically defined alterations in common, namely a particular rearranged *c-abl/bcr* region. In turn, although *c-abl/bcr* rearrangement appears to define CML cells, immunological techniques and additional probes for other genetic sequences such as the TCR or the immunoglobulin genes are required for detailed evaluation of the differentiation stage of CML blasts.

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Mediastinal Lymphoma of Clear Cell Type is a Tumor Corresponding to Terminal Steps of B Cell Differentiation

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This article reports eight primary mediastinal tumors occurring in young adults (19 to 43 years, mean 29.4 years), predominantly female (six of eight) adults. Most patients responded badly to aggressive therapy. Progression is presently noted in one patient; five patients died 10, 11, 13, 18, and 22 months after diagnosis. No patient developed leukemia. The tumors were highly proliferative, had a diffuse growth pattern, and comprised clear cells of variable size. They could not be classified histologically, but could, however, be immunohistologically characterized as B cell lymphomas. In all cases, the immunophenotype was LC$^+$, cALLa$^-$, CD19$^+$, CD20$^+$, CD21$^-$, Ig (surface/cytoplasm)$^-$, and PC-1$^+$. In addition, the neoplastic cells exhibited variable defects in the expression of HLA-A,B,C and HLA-DR and inconstant expression of other B cell-restricted/associated antigens. This combination of immunophenotypical and clinical features suggests that the mediastinal clear cell lymphoma (MCCL) is a previously undescribed type of B cell lymphoma corresponding to the terminal steps of B cell differentiation.

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NORMAL PERIPHERAL B cells express major histocompatibility (MHC) antigens of class I (HLA-A,B,C) and class II (HLA-D), lineage-restricted differentiation antigens CD19, CD20, CD22 (reviewed by Nadler[1]) and carry immunoglobulin (Ig) on their surface. At the terminal stages of differentiation, the B cell differentiation antigens, HLA-D, and surface Ig disappear; plasma cell-associated antigens such as the PC-1, PCA-1,[2,3] and the T10 antigen[4] are expressed, and Ig is secreted in large amounts. In neoplastic B cells, however, deficient Ig expression may occur more frequently than initially assumed since most of the so-called "null"-cell lymphomas and common acute lymphoblastic leukemias are actually of B cell origin[5-10]; even chronic B lymphocytic leukemia is reported to lack detectable Ig constituents in a small percentage of cases.[11] Apart from Ig deficiency, cytomorphology of B cell tumors may be so deviant from that of B cells in physiologic development that they occasionally mimic T cell lymphomas,[12-15] Hodgkin's disease,[16] and even carcinoma.[17] The mediastinum is known to be the primary site of lymphoblastic T cell tumors ("T cell, convoluted" according to Lukes and Collins[18]), Hodgkin's disease,[19] and epithelial malignomas such as thymic carcinoma, and rarely of sarcoma (reviewed by Otto[20]). Even large series of malignant lymphomas, however, (eg, Brittinger and colleagues,[21] 1,127 cases; Tubbs and colleagues,[22] 564 cases), of mediastinal lymphomas (Bernard and colleagues,[23] 50 cases) and extranodal B cell lymphomas (Mohri,[24] 148 cases) fail to contain primary mediastinal tumors of proven B cell origin. Nevertheless, there are three reports on primary non-Hodgkin's lymphoma of the mediastinum[25-27] with pronounced aggressiveness and poor prognosis. Conspicuously, these lymphomas have a number of features in common: The patients were mostly young female adults presenting cough, chest pain, dyspnea, and venous obstruction as initial symptoms. The tumors apparently originating in the mediastinum tended to invade adjacent organs and structures and in most cases responded badly to antineoplastic treatment. As to morphology, the tumors were described as being of diffuse large cell type or as poorly differentiated, lymphocytic. In one series,[27] pronounced sclerosis was described as an additional characteristic feature. Concerning the immunophenotype, Perrone and colleagues[27] were able to prove the "lymphoreticular origin" of 53 of their 60 mediastinal tumors by applying a mixture of monoclonal antibodies to leukocyte common (LC) antigen on paraffin sections. Furthermore, immunoreactivity with antisera to κ and λ light chains suggested a B cell origin of five of these tumors. The remaining tumors described by the three groups of authors could be characterized, however, neither as B nor as T cell lymphomas using surface marker techniques available at the time of publication or by using formalin-fixed material.

Here we report that these mediastinal "null"-cell lymphomas are of B cell origin and present an immunophenotype that, although characterized by various defects, is indicative of a very late stage of B cell maturation.

MATERIALS AND METHODS

Patients. During the last 2 years, eight unusual primary mediastinal tumors were observed. They occurred in young (19 to 43 years, mean 29.4 years) predominantly female (6 of 8) adults. First clinical symptoms consisted of thoracic pain, thoracic venectasia, and dyspnea; B symptoms were noted in only 1 case. Tumor localization was described as mediastinal in 6 and as (para-)thymic in 2 cases. Initial diagnosis revealed involvement of the lungs in 3, of the pericardium in 2, and of cervical lymph nodes in 1 case; venous compression or invasion was observed in 3 patients. None was initially leukemic or developed leukemia. Serum electrophoreses were normal. The clinical course was characterized by therapeutic failure. Even though aggressive protocols against high-grade malignant lymphomas were

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Table 1. Clinical Data

Case No.	Patient	Sex	Age at Diagnosis (years)	First Clinical Symptoms	Primary Localization	Tumor Cell Morphology	Preliminary Diagnosis*	Clinical Course After Surgical Intervention, Radiation and Chemotherapy
1	B.M.	F	19	Left breast venectasies; facial edema	(Para-) thymic	Large, polymorphic	Hodgkin's disease	Progression; liver infiltrations and abdominal lymphomas; tracheo-esophageal fistula; pneumonia; death 22 months after diagnosis
2	F.M.	F	21	B-symptoms: fever, night sweats, weight loss	Mediastinum	Large, polymorphic	Malignant lymphoma	Local progression; death 10 months after diagnosis with lung involvement and pericardic effusion
3	W.I.	F	31	Thoracic pain (left); dry cough	Mediastinum	Large and medium-sized, polymorphic	Malignant lymphoma	Local progression; death due to respiratory insufficiency 11 months after diagnosis
4	L.I.	F	35	Painful right shoulder/thorax; moderate dyspnea on exertion	Mediastinum	Relatively small, monomorphic	Malignant lymphoma	Local progression; abdominal lymph node involvement
5	G.S.	F	35	Thoracic pain, dry cough, moderate dyspnea on exertion	Mediastinum	Medium-sized to relatively small, monomorphic	Thymoma	Local progression; lung involvement; retroperitoneal lymphomas; death due to respiratory insufficiency 12 months after diagnosis
6	S.S.	F	43	Thoracic venectasies with cervical intumescence	Mediastinum	Medium-sized, monomorphic	Malignant lymphoma	Local progression; death due to respiratory insufficiency 18 months after diagnosis
7	K.M.	M	22	Mediastinal enlargement found in a routine X-ray	Mediastinum	Medium-sized, polymorphic	Hodgkin's disease	Presently in complete remission after combination chemotherapy
8	S.G.	M	29	Cervical swelling; facial and brachial edema	(Para-) thymic	Medium-sized, monomorphic	Malignant lymphoma	Progression; liver, kidney, and adrenal gland infiltrations; cervical lymphomas

*Done on the basis of routine hematoxylin and eosin sections for rapid notification.

applied in 7 cases, remission was achieved in only 1 case (patient 7, who received combination chemotherapy comprising endoxan, holoxan, vincristine, and prednisone [CHOP]). Five patients died 10, 11, 13, 18 and 22 months after initial diagnosis of extensive disease and large thoracic tumor masses causing respiratory insufficiency (Table 1).

Tumor tissue. Fresh tissue was transferred to our laboratory immediately after surgical removal. Representative samples were quick-frozen in liquid nitrogen. Frozen sections (4 to 6 μm) were air-dried overnight, fixed in acetone for 10 minutes at room temperature, and immunostained immediately or stored at −20°C for 1 to 3 weeks. Another sample was fixed in Bouin's fixative for routine processing purposes; histochemical stains from the paraffin-embedded material were achieved by using hematoxylin and eosin (H & E), periodic-acid Schiff's (PAS), Giemsa, and the Gomöri reticulin method; immunostaining of paraffin sections was preceded by bleaching and destruction of endogenous peroxidase by methanol/H_2O_2.

Reagents. Monoclonal antibodies (MAbs) to IgM (R1/69) and IgD (IgD26), to the leukocyte common antigen (2B11 and PD7/26), and anti–Ki-1 were obtained from Dakopatts, Copenhagen. MAbs against the T4 antigen (anti–Leu-3a (SK2)), the T8 antigen (anti–Leu-2b (SK3)), κ (163-42) and λ (1-155-2) light chains, to the interleukin-2 (IL2) receptor (2A3) and the CD22 antibody anti–Leu-14 were purchased from Becton Dickinson, Mountain View, CA. MAbs to the T11, T6, and T3 antigens were obtained from New England Nuclear, Seattle (Lyt3) and Ortho Diagnostics, Raritan, NJ (OKT6, OKT3), respectively. MAbs to common-ALL antigen (cALLa) (J5), to the B cell antigens CD20 (B1), CD21 (B2), CD19 (B4), and mAbs PC-1 and PCA-1, both directed against plasma cell-associated antigens, were furnished by Coulter Immunology, Hialeah, FL. MAbs specific to framework determinants of HLA-A,B,C (W6/32) and HLA-DR (2.06), and Blast-2, reacting with a B cell activation antigen (CD23 antigen) were generous gifts from the producing laboratories (given in references in Table 2); the B cell reagents HD6, HD28, HD37, HD237, HD39, and HD50 are products of some of us (B.D., G.M., S.K.), as is the antibody HEA125 that reacts with a surface glycoprotein (Egp34) restricted to normal and neoplastic epithelial cells (G.M., F.M.).

A polyclonal biotinylated sheep antibody to mouse Ig and a streptavidin-biotinylated peroxidase complex, both obtained from Amersham Buchler, Braunschweig, FRG, served as detection system for the monoclonal primary antibodies. Unconjugated rabbit-derived antisera to human μ-, γ-, α-, ϵ-, κ-, and λ-chains, swine anti-rabbit Ig serum and peroxidase–rabbit antiperoxidase complex were purchased from Dakopatts. Anti-human δ-chain and J-chain sera were obtained from Nordic Immunology, Tilburg, Netherlands. Pooled human IgG (Gamma-Venin) was supplied by Behringwerke, Frankfurt, FRG, 3-amino-9-ethyl carbazole (AEC) and *N'N*-dimethylformamide (DMF) were obtained from Sigma, St Louis.

Staining procedures. MAbs in culture supernatants were used undiluted; ascites preparations were used as 1:200 dilutions. The anti-mouse Ig antibody was diluted 1:50, and the streptavidin–peroxidase complex was diluted 1:100. Dilutions of primary antisera ranged from 1:50 to 1:1500; the anti-rabbit Ig antiserum was diluted 1:20, and the peroxidase–antiperoxidase complex was diluted 1:100. All dilutions and washing steps were carried out in phosphate-buffered saline (PBS); the secondary antibody solution contained 5% pooled human IgG to inhibit cross-reactions with human surface Ig. Incubation times of tissues were 1 hour at room temperature for the primary antibody (respective antiserum) and 30 minutes for the second-step and third-step reagents. Using AEC as the chromogen (0.4 mg/mL in 0.1 mol/L of acetate buffer pH 5.0 with 5% DMF and 0.01% H_2O_2 for 10 minutes), the peroxidase reaction resulted in

Table 2. Antigens Detected and Primary Antibodies Used in This Study

Antigen	MW KD	Clone	Source (Reference)
MHC			
HLA-A, B, C	12;45	W6/32	Barnstable et al (28)
HLA-DR	29;34	2.06	Charron and McDevitt (29)
B cell-associated antigens			
Leukocyte common	200	PD7/26, 2B11	Warnke et al (30)
cALLa (CD10)	100	J5	Ritz et al (31)
B cell-restricted antigens			
CD 19	95	HD37	Pezzutto et al (32)
		B4	Nadler et al (33)
CD 20	35	B1	Stashenko et al (34)
CD 21	140	B2	Nadler et al (35)
CD 22	135	HD6	Moldenhauer et al (36)
		HD39	Dörken et al (37)
		Leu14	Schwarting et al (38)
CD 23	45	Blast-2	Thorley-Lawson et al (39)
		HD50	(author's laboratory)
Plasma cell-associated antigens			
Plasma-cell antigen	28	PC-1	Anderson et al (2)
Plasma-cell antigen	24	PCA-1	Anderson et al (3)
T10 antigen	45	OKT10	Reinherz et al (4)
Varia			
Ki-1 antigen	116/126	Ki-1	Stein et al (40)
IL2 receptor	60	2A3	Pali et al (unpublished observations) (Becton Dickinson)
T cell antigens			
T11	55	9.6	Kamoun et al (41)
T6	49	OKT6	Reinherz et al (4)
T3	19	OKT3	Reinherz et al (42)
T4	55	SK2	Evans et al (43)
T8	32;43	SK3	Evans et al (43)
Epithelial antigen			
Egp34	34	HEA125	Momberg et al (44)

MHC, major histocompatibility complex.

an intense red precipitate. The sections were counterstained with Harris' hematoxylin and mounted with glycerol gelatin.

Controls. To obtain reliable results, the detection of Ig components was performed twice, using antisera on paraffin sections and mAbs on frozen sections. Positive controls were made at the same time, using paraffin and frozen sections from tonsils to ensure that a negative result on the tumor tissue was reliable and not due to the method. Intrinsic positive controls for the immunoreactivity of the MHC antibodies were interstitial dendritic cells, endothelial cells, and macrophages that, by their staining, indicated the reliability of the reaction and thus excluded false-negative results. Negative controls were performed by omitting the primary antibody (antiserum). No staining was observed, except for the reaction of intermingled granulocytes in frozen sections whose endogenous peroxidase was not destroyed.

RESULTS

Histomorphology. Most of the tumors showed a diffuse growth pattern (Fig 1). The cells had clear and abundant cytoplasm in common but varied considerably in size. The nuclei were roundish to very irregular; one tumor contained cells with multilobated nuclei, and a second revealed giant cells with extremely pleomorphic nuclei resembling Reed-Sternberg cells. The nucleoli were predominantly small and mostly singular. In cases 1 and 2, the tumor cells are best described as large and pleomorphic; in case 3 as mixed, large and medium-sized and pleomorphic; in cases 6 and 8 as medium-sized monomorphic; and in case 5 (Fig 1) as mixed, medium-sized to relatively small but monomorphic. The smallest tumor cells, however, were twice as large as the diffusely infiltrating normal lymphocytes that were a regular constituent of the lesion. Tumor necroses, mostly ill-defined and small but never confluent, were observed in six cases. Mitoses were numerous and always typical. Even more frequent were single-cell degenerations. The tumors were further characterized by an alveolar to microalveolar reticulin fiber scaffold; case 1 showed pronounced sclerosis. In conclusion, the histologic picture of these tumors was not compatible with any of the morphologically defined B cell lymphomas listed in the current classifications. The histomorphology of these lymphomas resembles either pleomorphic peripheral T cell lymphoma or thymoma; in two cases, it even resembled Hodgkin's disease.

Immunophenotype. (Table 3) The tumors were LC$^+$ and HEA125$^-$ and thus clearly defined as nonepithelial in nature. They all expressed the B cell-restricted antigens CD19 and CD20, as could be shown by the binding of mAbs HD37, HD237, B4, and B1, respectively. The first three mAbs showed minor differences in binding intensity in five cases; B4 failed to react with tumors 3 and 7. CD22 was expressed on the tumor cells of six cases but was undetectable

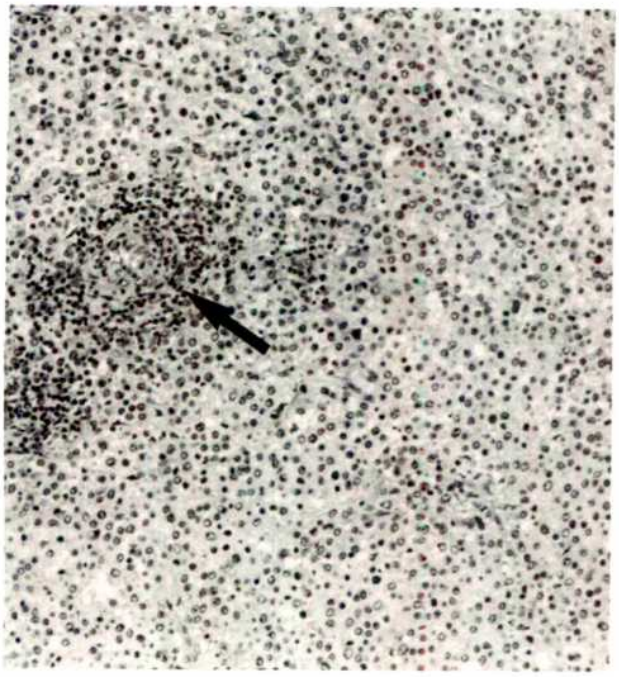

Fig 1. Primary mediastinal clear cell lymphoma of B cell type: Case 5. Predominantly medium-sized tumor cells with very pale cytoplasm and roundish nuclei, diffuse growth pattern. Arrow: vessel surrounded by normal peripheral (T) lymphocytes. Hematoxylin and eosin; original magnification ×140.

in cases 3 and 8. CD23, a putative B cell activation antigen, was present on tumor cells of cases 1 and 4 (Fig 2) and on a minor part of the neoplastic cells of cases 7 and 8. Two other activation antigens, Ki-1 and the IL 2 receptor, could not be detected. Immunoglobulin constituents (heavy and light chains, J chain) were not detectable in the whole tumor series in either the cytoplasm or on the surface of the tumor cells. Ig of various light and heavy chain classes was present only in mature, morphologically typical plasma cells, which, however, were very scarce. In addition, one case contained minute islands of small, round lymphoid cells carrying surface IgM (Fig 3). The neoplastic cells of every case strongly bound PC-1, a mAb recognizing a plasma cell-associated antigen (Fig 4). PCA-1, another mAb of this category, reacted in cases 5 and 6 with the entire neoplastic population and in a varying number of tumor cells of cases 1, 2, and 4. MAb OKT10, also known to bind to normal plasma cells, reacted with tumor 2 and with parts of the neoplastic population of case 4 CALLA and CD21 could not be detected in either case. The tumors showed variable deficiencies in MHC antigen expression: at least two cases completely lacked class I and class II antigens (Fig 5); a third case (case 6) contained only a very small number of scattered HLA-ABC$^+$ and HLA-DR$^+$ large cells among the unreactive tumor cells (Fig 6); although probably of histiocytic origin, it cannot be completely ruled out that those cells were part of the neoplastic population. Four other tumors contained varying proportions of MHC antigen-deficient neoplastic cells. The coordinate expression of class I and class II

Table 3. Antigen-Expression of Tumor Cells in Eight Cases of Ig-Mediastinal Clear Cell Lymphoma of B Cell Type

Antigen	Clone	Patient							
		1, B.M.	2, F.M.	3, W.I.	4, L.I.	5, G.S.	6, S.S.	7, K.M.	8, S.G.
HLA-ABC	W6/32	•−	+	−	±	−	− ≫ +	−	±
HLA-DR	2.06	±	+	−	+ > −	+ > −	− ≫ +	−	+
LC	PD7/26 and 2B11	+	+	+	+	+	+	+	+
CALLA (CD 10)	J5	−	−	−	−	−	−	−	−
CD 19	HD37	+	+	(+)	+	+	+	+	(+)
	HD237	(+)	+	±	+	+	+	+	(+)
	B4	+	+	−	+	(+)	+	−	(+)
CD 20	B1	+	+	+	+	+	+	+	+
CD 21	B2	−	−	−	−	−	−	−	−
CD 22	HD6	+	+	−	+	(+)	+	+	−
	HD39	+	+	−	+	+	+	+	−
	Leu14	+	+	−	+	+	+	(+)	−
CD 23	Blast-2	+	−	−	+	−	−	±	±
	HD50	+	−	−	+	−	−	−	−
Plasma cell-associated antigen	PC-1	+	+	+	+	+	+	+	+
	PCA-1	+	−	−	− ≫ +	−	−	−	−
	OKT10	−	+	−	− > +	−	−	−	−
Ig (cytoplasmic/surface)		−	−	−	−	−	−	−	−
Ki-1 antigen	Ki-1	−	−	−	−	−	−	−	−
IL2 receptor	2A3	−	−	−	−	−	−	−	−
T cell antigens T6, T11, T3, T4, T8	OKT6, Lyt3, OKT3, Leu3a, Leu2b	−	−	−	−	−	−	−	−
Egp34	HEA125	−	−	−	−	−	−	−	−

•−, Negative; +, positive reaction on the entire tumor cell population; (+), weakly positive; ±, positive and negative tumor cells in equal parts, +, negative tumor cells clearly outnumber positive ones; − ≫ +, very few scattered positive cells, putative tumor cells.

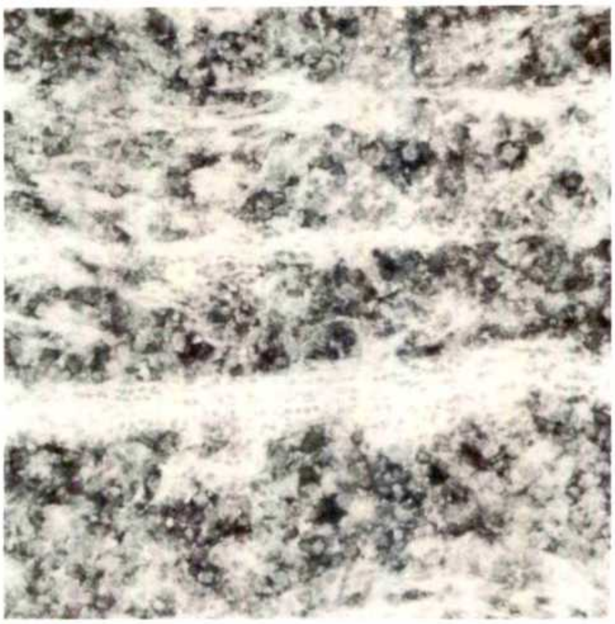

Fig 2. Case 4. Monoclonal antibody (MAb) HD50 recognizing the activation-associated CD23 antigen bound in varying intensity to the tumor cell population but not to the stromal cell component of the lymphoma. Immunoperoxidase; original magnification ×122.

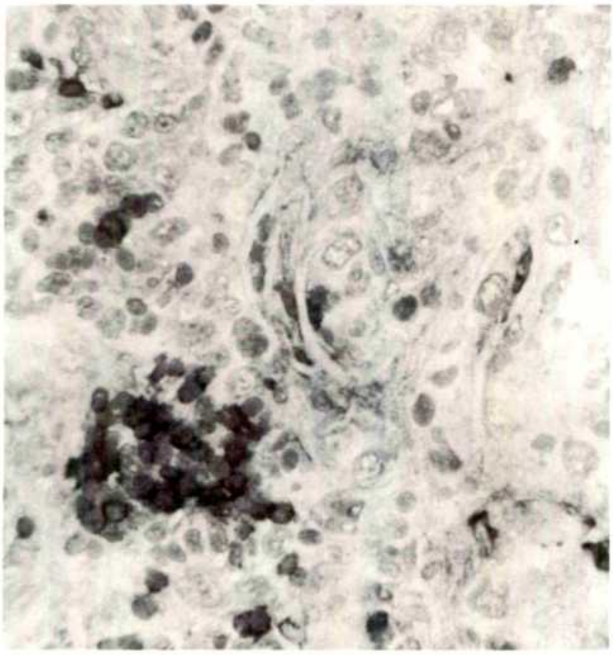

Fig 3. Case 2. Demonstration of IgM by monoclonal antibody (MAb) R1/69. The large tumor cells were devoid of detectable IgM whereas scattered reactive small lymphocytes stained strongly, thus excluding a false-negative result. Immunoperoxidase; original magnification ×305.

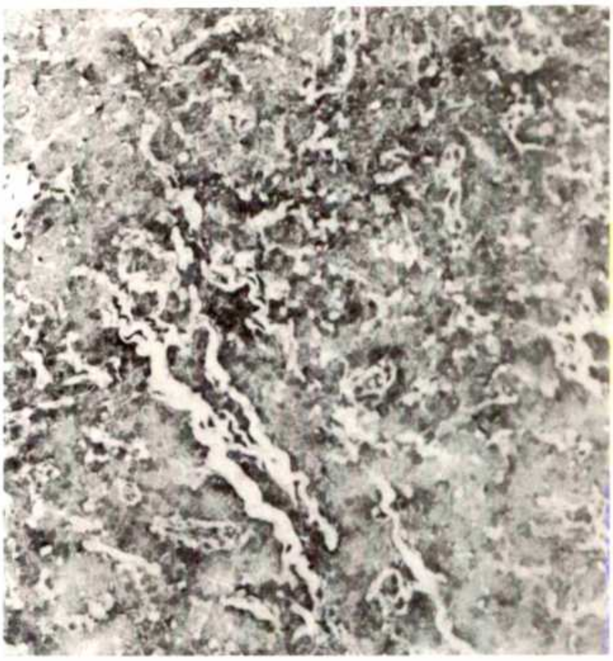

Fig 4. Case 8. Monclonal antibody (MAb) PC-1 recognizing a plasma cell-associated antigen bound strongly to the entire tumor cell population. Negative connective tissue fibers are apparent. Immunoperoxidase; original magnification ×133.

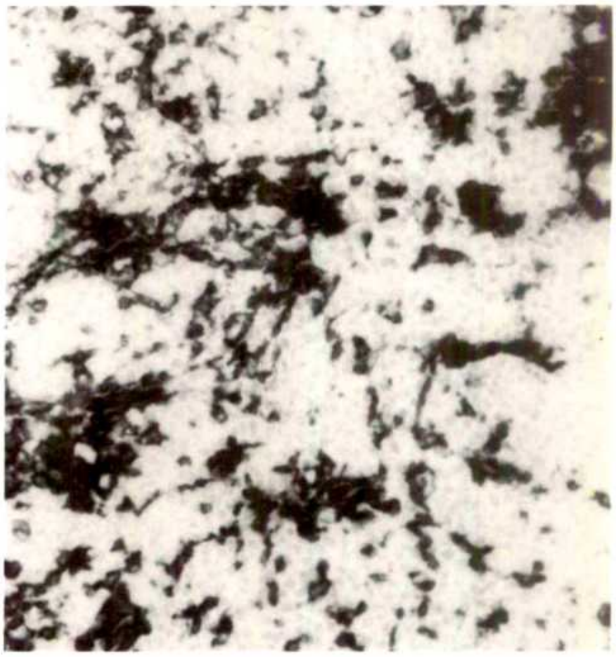

Fig 5. Case 5. Monoclonal antibody (MAb) W6/32 strongly stained dendritic interstitial cells and small infiltrating lymphocytes but did not bind to the tumor cell population, thus indicating its abnormal loss of HLA-A,B,C molecules. Immunoperoxidase; original magnification ×122.

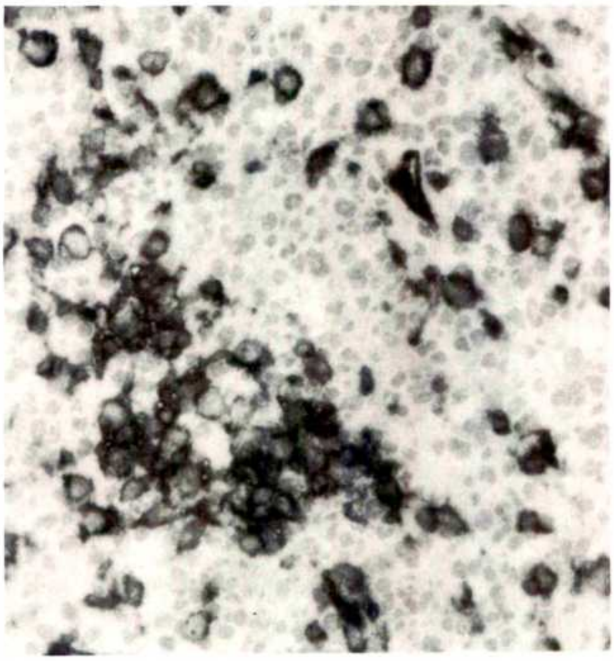

Fig 6. Case 6. Monoclonal antibody (MAb) 2.06 recognizing a HLA-DR framework determinant failed to react with the tumor cells but stained interstitial dendritic cells and a small proportion of large scattered cells, most probably of histiocytic origin. Immunoperoxidase; original magnification ×195.

antigens was noticed only in case 2. MAbs directed against T cell antigens T6, T11, T3, T4, and T8 exclusively stained small lymphoid cells interspersed in various amounts with the tumor cells and occasionally crowded around blood vessels (Fig 1). The epithelium-specific mAb HEA125 showed reactivity in neither case.

In sum, this series of eight polymorphic mediastinal tumors is characterized by variable defects in MHC antigen expression, by the constant phenotype $CD10^-$, $CD19^+$, $CD20^+$, $CD21^-$, $PC\text{-}1^+$ and complete Ig deficiency, suggesting that they represent tumors of terminal B lymphocyte differentiation.

DISCUSSION

In several aspects, the clinical features of our patients with primary non-Hodgkin's lymphoma of the mediastinum resemble those of the patients described by Levitt and colleagues,[25] Trump and Mann,[26] and Perrone and colleagues.[27] As compared with mediastinal T lymphoblastic lymphoma, which has pronounced male predominance (eg, Brittinger and colleagues,[21] ratio of males to females 9:1), there seems to be a clear female predominance for mediastinal "null"-cell lymphoma: 6 of 12 of the patients of Levitt and colleagues, 7 of 11 of the patients of Trump and Mann, 43 of 60 of the patients of Perrone and colleagues and 6 of 8 of those in our series were female. Nine of those of Levitt and colleagues, 8 of Trump and Mann's, and 7 of our patients were younger adults (19 to 37 years); 85% of Perrone and colleagues' group were aged ≤35 years. Cough, chest pain, dyspnea, and venous obstruction were common initial signs. Apart from mediastinal mass, there was additional tumor involvement of pericardium at initial examination in three of the patients of Levitt and colleagues, 1 of the patients of Trump and Mann, and in 2 of the patients of our series; lung involvement was noted twice in Levitt and colleagues' group, 3 times in Trump and Mann's group, and 3 times in our group of patients. The chest wall was the most frequent site of extrathoracic involvement in Perrone's series. When given aggressive antineoplastic treatment, 10 of the patients of Levitt and colleagues reached a transient complete remission, but neither of Trump and Mann's patients and only one of our patients was regarded as disease-free afterwards. No patient developed leukemia. Progressive disease, relapse, and chemotherapeutic failure was commonly noted. Four of the patients of Levitt and colleagues, 8 of Trump and Mann's, 20 of the patients of Perrone and colleagues, and 5 of our patients were dead at time of publication, with a survival time ranging from 4 to 22 months; median survival given by Trump and Mann was 16 months. To conclude, we feel that we are dealing with a lymphoma type with a rather distinct clinical picture.

Our central finding is that these tumors are B cell lymphomas. Referring to the B cell differentiation scheme given by Nadler,[1] the immunophenotype LC^+, $CD10^-$, $CD19^+$, $CD20^+$, $CD21^-$, ($Ki1^-$, T cell antigens$^-$), common to all the tumors of our series, suggests a terminal B cell differentiation. This view is supported by the presence of the plasma cell-associated antigens PC-1 in 8 of 8, PCA-1 in 5 of 8, and T10 in 2 of 8 tumors, indicating that at least a partial plasma cell phenotype is expressed although cytomorphology is never plasmocytic. On the basis of the PCA-1 reactivity of neoplastic cells, Anderson and co-workers[45] characterize hairy cell leukemia as a tumor of the pre-plasma cell. Plasmacytoma, plasma cell leukemia, and myeloma are reported to express PCA-1,[45] PC-1,[2] and (OK)T10.[46,47] On the other hand, the diffuse large cell lymphomas of B cell type analyzed by Freedmann and co-workers[48] failed to express PCA-1 and PC-1, a finding that caused them to argue that these tumors are the neoplastic counterparts of normal B cells at the midstage of differentiation. Based on these observations the immunophenotype of the aleukemic lymphomas described herein indicates that it is probably a novel B lymphoma type. It might be placed between tumors of midstage differentation and hairy cell leukemia/plasma cell tumors. This tumor cell variant might alternatively be regarded as the malignant counterpart of the final stage of an alternative pathway of B cell development: the monocytoid B cell, also known as the cell of the so-called "immature sinus histiocytosis,"[49-52] which is a B cell that cannot be located in the current physiological differentiation scheme. In this context, it is noteworthy that monocytoid B cells have clear cell morphology and are $PCA\text{-}1^+$ (P. Möller, unpublished observations).

Nevertheless, as compared with the normal mature B cell, the tumor cells of MCCL exhibit considerable lacks in antigen expression. There are no Ig constituents. This complete loss of Ig has been observed by several authors (discussed previously) to occur in large cell B lymphomas of unknown or follicular origin. Two of our tumors failed to express the CD22 antigen, as could be verified by staining with three different mAbs. This may be interpreted in two ways. CD22 expression may either have been impaired by a defect in gene expression or abrogated in the course of terminal differentiation. Furthermore, there are minor irregularities in the binding of CD19 mAbs: HD237 failed to react with a subset of neoplastic cells of case 3; B4 did not recognize tumors 3 and 7. Whether this is due to inaccessibility or defect of the corresponding epitope or just to a lesser affinity of these antibodies cannot be answered. Because the CD23 antigen seems to be closely related to activation,[39] the partial or complete nonexpression of the CD23 antigen in six of eight tumors is probably of functional nature. Severe defects, however, must be suspected because of the partial or complete nonexpression of MHC class I molecule (HLA-A,B,C) observed in all tumors but one. To our knowledge, very few reports exist describing lack of class I antigen expression in malignant lymphoma: Woda and co-workers[53] observed this lack in three cases of large "histiocytic" B cell lymphomas, and the Daudi cell line is known to be devoid of β_2-microglobulin,[54] which is part of the heterodimeric class I molecule. Because class I antigens serve as restriction elements of T cell-mediated cytotoxicity,[55] HLA-A,B,C-deficient tumor cells possibly escape immune attack by cytotoxic T cells.[56] This may account for the clinical aggressiveness of the primary mediastinal B cell lymphomas.

Six of eight of the tumors had either partial or complete defects in expression of the HLA-DR framework determinant recognized by mAb 2.06. Defects in class II antigen expression in B cell lymphomas were sporadically noted by several authors.[57-61] Our evaluation of the staining for HLA-DR probably underestimates the real extent of nonreactivity within the neoplastic population since intermingled small negative cells were regarded as reactive T cells, as was suggested by parallel sections stained with mAbs to T cell antigens. For multiple myeloma, it was assumed that the neoplastic plasma cells lost their class II antigens while entering the terminal stage of differentiation.[45-62] Although we discuss this stage of development for our tumor group, a down-regulation of MHC class II antigen expression seems more unlikely to us than assumption of a pathological loss, ie, a further defect in antigen expression since all tumors lacking class II molecules were also class I antigen-deficient. Class II antigens are transducers for B cell activation[63-65] and maturation.[66] Thus, a defective expression of these important trigger molecules might contribute to the incomplete differentiation of the neoplastic B cells in the primary mediastinal lymphoma of clear cell type.

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G-150 for gel filtration. The C1̄-INH prepared by this method was usually a homogeneous single band in SDS analytic gel electrophoresis, but some preparations demonstrated two closely approximated bands.[8,9] Recoveries were as high as 70% of that in the starting plasma.

Plasma kallikrein was isolated according to the procedure of Nagase and Barrett,[10] which was modified to include a second step in affinity chromatography with SBTI-Sepharose following chromatography on Ultrogel AcA 54. In addition, immune adsorption on a column of Sepharose-antihuman IgG was used to remove contaminating immunoglobulins at the end of the purification. This kallikrein was homogeneous in SDS analytic gel electrophoresis when freshly prepared, or a doublet, and had a specific activity of approximately 176 units of coagulant activity per milligram of protein. In determining the specific activity of plasma kallikrein preparations, a coagulation assay was used[11] in which one unit of activity is defined arbitrarily as the amount in 1.0 mL of pooled normal human plasma. During storage at −30°C, autodigestion products formed of 67,000 and lower mol wt. Cleavage products of less than 52,000 mol wt, induced by cleavage by factor XIIa, have been shown to have reduced ratios of coagulation to amidolytic activity.[12]

C1s̄ was prepared by the method of Bing and his colleagues[13,14] and gave a homogeneous single band in SDS-analytic polyacrylamide gel electrophoresis when freshly prepared. This material had a specific activity of over 1,000 U/mg of protein.[1] Some degradation of the C1s̄ used has occurred in the present studies, providing lower mol wt bands visible in stained gels (see figures) after EDTA was removed during gel filtration.

SDS-polyacrylamide gel analyses were performed using a Hoefer Vertical Gel Electrophoresis system and was carried out either in 7.5% polyacrylamide, according to the method of Laemmli[15] or in gradient gels prepared using a modification of this method in which a gradient of 7.5% to 12.5% (wt/vol) acrylamide was prepared as reported previously.[8] Gel-bond (FMC, Marine Colloids Division, BioProducts, Rockland, ME) was inserted between the glass plates into which the gels were poured for vertical electrophoresis to stabilize the gels. After adding SDS, the mixtures were incubated at 37°C for another 30 minutes, instead of boiling, to avoid aggregation of proteins. Electrophoresis was carried out at 3 to 3.5 V/cm for 18 hours at room temperature or until the tracking dye was within 1 to 2 cm of the bottom of the gel when gradient polyacrylamide gel was used. When a single concentration of polyacrylamide gel was used, electrophoresis was carried out at 4°C until the tracking dye was near the bottom of the gel. After staining with 0.25% Coomassie brilliant blue, the gels were fixed in mixtures of 33% methanol, 15% trichloroacetic acid, and 3% sulfosalicylic acid for 30 minutes, followed by washing in an aqueous mixture of 5% acetic acid and 16% glycerol overnight.

RESULTS

Interactions of C1s̄ and plasma kallikrein with normal C1̄-INH. When purified normal C1̄-INH was incubated with plasma kallikrein for as short a period as five minutes at 37°C, stable complexes between kallikrein and the inhibitor formed (Fig 1). These complexes appear to involve the component of C1̄-INH of about 106,000 mol wt but not that of approximately 96,000 mol wt, even when the concentration of kallirein was in molar excess with respect to the inhibitor (Fig 1A). In experiments not shown, complexes between the *lower* mol wt component of C1̄-INH and kallikrein in equimolar amounts or in enzyme excess failed to

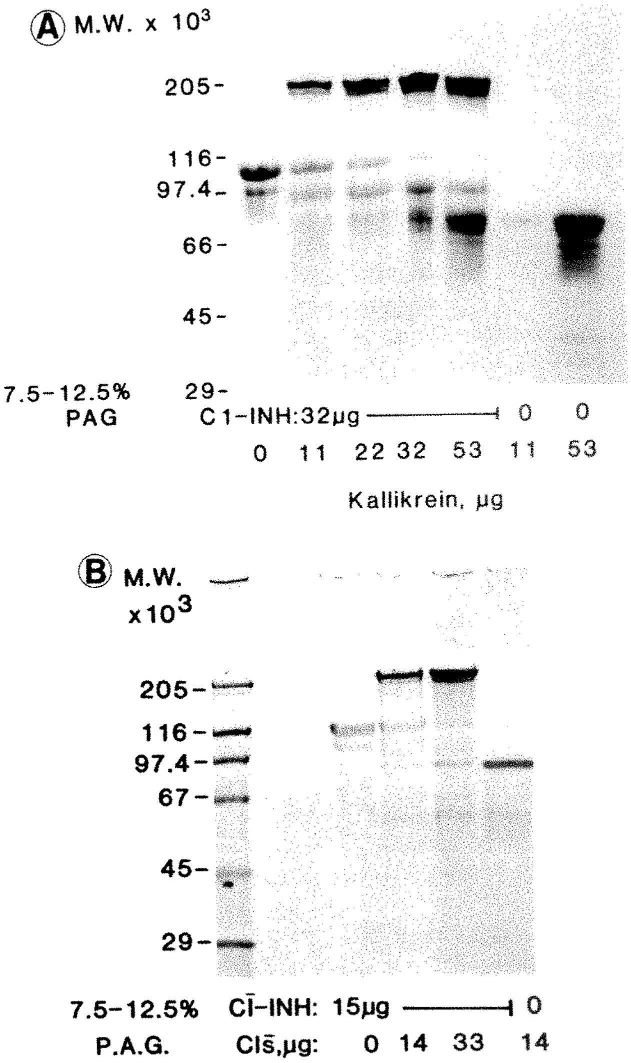

Fig 1. (A) Variable amounts of purified plasma kallikrein were incubated with 32 µg of purified normal C1̄-inhibitor at 37°C for 20 minutes. SDS was added, the mixtures incubated at 37°C for another 30 minutes, and were then subjected to electrophoresis in a vertical thin slab of polyacrylamide gel in graded concentrations of 7.5% to 12.5%. The sample in the left lane (not shown) was a mixture of proteins of known mol wt, noted to the left. The sample in the lane to the far right was plasma kallikrein alone (K), which was partially degraded. Note that the lower mol wt form of C1̄-INH was not consumed in complexes with kallikrein, even in enzyme excess (lanes 2 to 5) and that this band assumes a position of lower mol wt materials after exposure to kallikrein than in the "native" state (lane 1). (B) The same preparation of purified C1̄-INH was incubated with increasing amounts (left to right) of C1s̄ at 37°C for 20 minutes before SDS was added and incubation continued for an additional 30 minutes at 37°C. Samples were then subjected to electrophoresis in thin slab vertical gels consisting of graded concentrations of polyacrylamide from 7.5% (top) to 12.5% (bottom, anode) as above. The positions of bands of proteins of known mol wt are indicated at the left. Note that both the higher and lower mol wt components of C1̄-INH appear to participate in complex formation with C1s̄ (lanes 3 and 4). The lower mol wt component appears to have been cleaved by C1s̄ (lane 3); this cleavage was not visible in ungraded gels. The apparent mol wt of the individual proteins is higher in this gradient gel system than in a continuous concentration of polyacrylamide.

occur during incubation for as long as two hours. On the other hand, when C$1\bar{s}$ was incubated with this preparation of C$\bar{1}$-INH, both components of the C$\bar{1}$-INH appeared to become involved in complexes with C$1\bar{s}$ (Fig 1B). These observations suggest that there are different structural requirements for the formation of stable complexes between normal C$\bar{1}$-INH and kallikrein than with C$1\bar{s}$.

When a purified preparation of normal C$\bar{1}$-INH, which was apparently homogeneous having a mol wt of 106,000, was used, it was cleaved by both C$1\bar{s}$ and kallikrein during incubation for brief periods at 37°C (Fig 2). The cleavage product had an approximate mol wt of 96,000. A major high mol wt complex formed with each enzyme and this inhibitor. The mol wt of the complex with C$1\bar{s}$ was approximately 182,000 and that with kallikrein was 185,000 (Fig 2). The C$\bar{1}$-INH was not completely consumed in this experiment, probably because the C$1\bar{s}$ used had a lower specific activity than that used in the experiment shown in Fig 1B. In an experiment not shown here, a preparation of C$\bar{1}$-INH, which had lost most of its activity against C$1\bar{s}$, consisted of two lower mol wt components of approximately 95,000 and 92,000. Neither of these formed a complex with plasma kallikrein.

Interactions of plasma kallikrein with dysfunctional C$\bar{1}$-INH. Dysfunctional C$\bar{1}$-INH proteins from members of eight different kindred with type II HANE were purified and analyzed for their interactions with plasma kallikrein. The subjects from whom plasma was obtained were not receiving androgen therapy at the time, with the exception of one preparation from subject Da, noted below, and therefore did not have therapeutically induced increases in serum concentrations of normal C$\bar{1}$-INH. These proteins were examined for their interactions with C$1\bar{s}$ and plasmin in work reported earlier.[8] Table 1 summarizes the interactions of these proteins with kallikrein and compares them with their interactions with C$1\bar{s}$, most of which were reported in an earlier study.[8] In these studies the kallikrein and inhibitor proteins were incubated together at 37°C for 20 minutes before SDS was added, and electrophoresis was performed in SDS gels. The preparation of C$\bar{1}$-INH from subject Bo contained a single low mol wt contaminant, and C$\bar{1}$-INH from subject Ta contained a single unidentified high mol wt contaminant visible in SDS gel. The preparation of C$\bar{1}$-INH from Da, which formed a stable high mol wt complex with kallikrein, was obtained when this patient had received androgen therapy and probably contained detectable amounts of normal C$\bar{1}$-INH. It was not possible to distinguish the normal protein from some of the dysfunctional protein in these gels. Other preparations from this subject failed to form complexes stable in SDS gels.

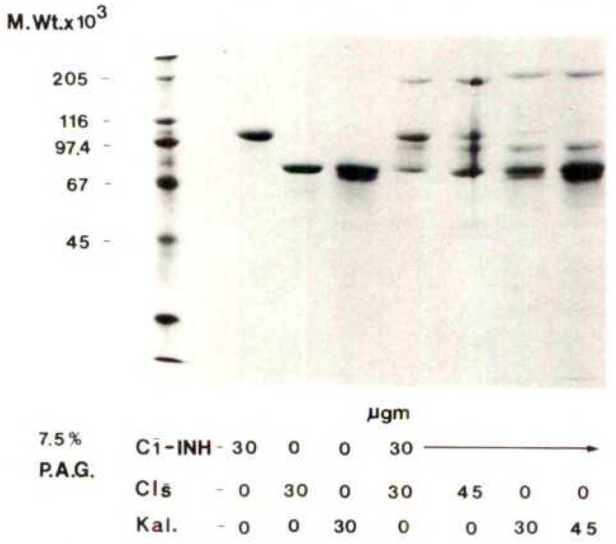

Fig 2. In this 7.5% polyacrylamide gel, the electrophoretic behavior of 30 µg of normal single band C$\bar{1}$-INH following incubation for 20 minutes with increasing amounts of C$1\bar{s}$ or kallikrein (µg), shown at the bottom, are compared. This C$\bar{1}$-INH was a preparation of high specific activity; the C$1\bar{s}$ was of lower specific activity than that used in Fig 1B. Mol wt standards are shown to the left and in lane 1; untreated C$\bar{1}$-INH alone is in lane 2, C$1\bar{s}$ alone in lane 3, and kallikrein alone in lane 4. The mixtures did not run at an identical rate in this gel (the running front was convex), but the calculated mol wt of the C$\bar{1}$-INH cleavage fragment generated by C$1\bar{s}$ was 96,000 and that generated by kallikrein was 94,000. Moreover, the C$\bar{1}$-INH-kallikrein complexes were of a higher mol wt (lanes 7 and 8) than the C$\bar{1}$-INH-C$1\bar{s}$ complexes (lanes 5 and 6).

In the experiment illustrated in Fig 3, kallikrein was incubated with the inhibitor proteins at 37°C, as above, and before electrophoresis SDS was added, and the mixtures were incubated at 37°C for 30 minutes, instead of boiling, to

Table 1. Interactions C$\bar{1}$-INH Proteins with C$1\bar{s}$ and Kallikrein

	Activity: % of Normal v		Complex Formation with		Cleavage by	
C$\bar{1}$-INH	C$1\bar{s}$	Kallikrein	C$1\bar{s}$	Kallikrein	C$1\bar{s}$	Kallikrein
At	50	2.5	+	+	+	0
Bo	61	16	+	0	0	+*
Da	75	50	+	0	+	+*
Mo	52	6	+	±	+	0
Ri	4	1	±	±*	+	0
Ta	90	10	+	0	+	+
We	19	33	+	0	+	+
Za	19	64	+	+	0	0
Normal:	100	100	+	+	+	+

The first column designates the abnormal C$\bar{1}$-INH from kindred with HANE by the first two letters of the kindred name, and the second and third columns provide the levels of inhibition of C$1\bar{s}$ and kallikrein by each dysfunctional C$\bar{1}$-INH as compared to that of the normal C$\bar{1}$-INH, which were published earlier.[8] The inhibition of C$1\bar{s}$ and kallikrein by normal C$\bar{1}$-INH were arbitrarily determined to be 100%. The percent of inhibition provided by each dysfunctional C$\bar{1}$-INH was calculated by dividing the reduction rate of hydrolysis of synthetic substrates by each enzyme, in nanomoles per minute per microgram of dysfunctional inhibitor, by the reduction in rate per minute per microgram of normal C$\bar{1}$-INH × 100. Each dysfunctional C$\bar{1}$-INH was tested four to six times against each enzyme using different quantities of inhibitor.[8] Kallikrein inhibition was measured with a synthetic chromogenic amide (S-2302, Kabi, Stockholm, Sweden) and inhibition of C$1\bar{s}$ by the method of Levy and Lepow,[1] which employs an acidometric titration of N-acetyl-L-tyrosine ethyl ester hydrolysis. The reduction in rate of esterolysis in the presence of the inhibitor proteins by a preparation of C$1\bar{s}$ in units per milligram of protein was calculated. Columns 4, 5, 6, and 7 indicate whether or not complex formation occurred between C$1\bar{s}$, kallikrein (+ = stable complexes) and each inhibitor and if the inhibitors were cleaved (+) by either enzyme.

*With molar excess of enzyme only (2:1).

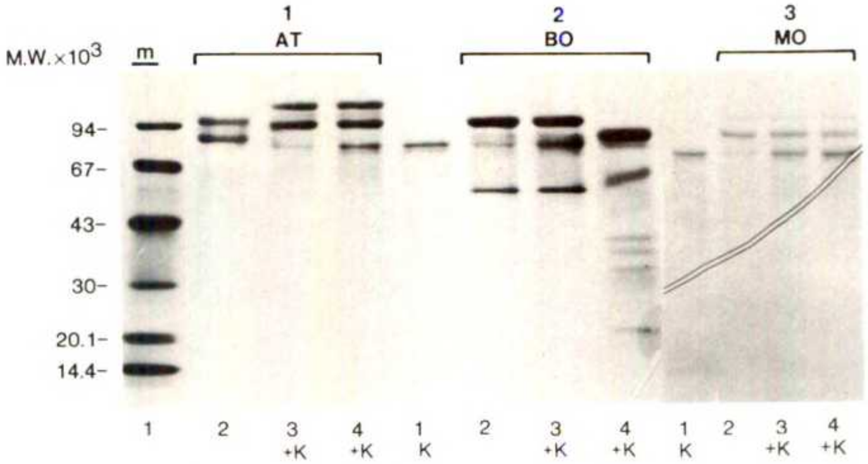

Fig 3. Each purified dysfunctional C$\bar{1}$-inhibitor protein is indicated by the first two letters of the kindred name, shown at the top of the gel, describing its electrophoretic behavior following preliminary incubation with one or more quantities of purified plasma kallikrein (K). Lane 1 (A) contained proteins (m) of known mol wt, indicated to the left. The gels contained concentrations of polyacrylamide graded continuously of from 7.5% at the top of the gel to 12.5% at the bottom (anode). The amounts of kallikrein and of C$\bar{1}$-INH proteins were: (A, B) C$\bar{1}$-INH At and Bo, 31 μg; kallikrein, 30 μg (lane 3) and 60 μg (lane 4); Lane 1, (B), kallikrein alone, 30 μg. (C): C$\bar{1}$-INH Mo, 28 μg (lanes 2 to 4); kallikrein, 30 μg (lane 3) and 60 μg (lanes 1 and 4). Although a somewhat higher mol wt band was generated in mixtures of C1-INH At and kallikrein, low mol wt fragments were not found. Possibly the complex contained a cleavage fragment of C1-INH At and kallikrein (panel 1). A low mol wt band in the C$\bar{1}$-INH Bo preparation (panel 2, lane 2) is an unidentified contaminant, reported earlier.[8] Although C$\bar{1}$-INH Bo failed to form a stable complex with kallikrein, it was cleaved by the enzyme (lane 4). There was no apparent interaction between C$\bar{1}$-INH Mo and kallikrein (panel 3).

avoid aggregation of proteins. After electrophoresis, C$\bar{1}$-INH from subject At appeared to form a lower mol wt complex than would be predicted with plasma kallikrein (Fig 3, lanes 3 and 4, panel 1). In contrast, C$\bar{1}$-INH from Bo was cleaved into multiple visible lower mol wt cleavage fragments during incubation with kallikrein but did not form a stable, higher mol wt complex (Fig 3, panel 2). C$\bar{1}$-INH from subject Mo apparently failed to interact with the kallikrein, even in excess (Fig 3, panel 3). C$\bar{1}$-INH Da also exhibited lower mol wt cleavage fragments when incubated with a molar excess of kallikrein (not shown). Thus under comparable conditions the dysfunctional proteins were heterogeneous in their susceptibility to cleavage by kallikrein. In earlier studies, C$\bar{1}$-INH We formed a complex with plasmin and was also readily cleaved by this protein.[8] This protein was also cleaved by plasma kallikrein but only into a single cleavage product of approximately 96,000 mol wt.

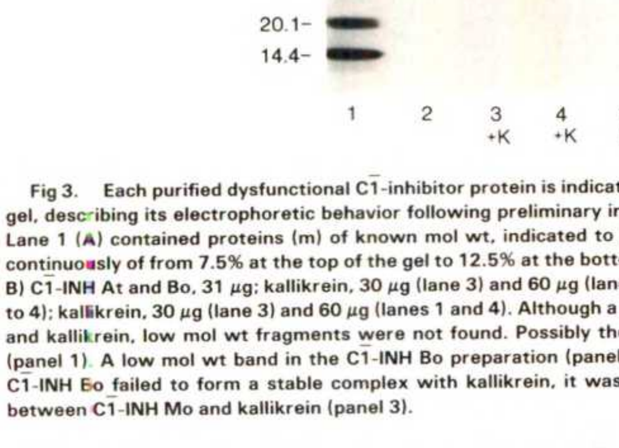

Fig 4. Comparison of electrophoretic behavior of dysfunctional C$\bar{1}$-INH At, Bo, and Za with C1$\bar{s}$ in SDS polyacrylamide gels of 7.5% concentrations (ungraded). C$\bar{1}$-INH was used in molar excess with respect to C1$\bar{s}$: C$\bar{1}$-INH At in an amount of 22.5 μg; C$\bar{1}$-INH Bo (lanes 4, 5), 25 μg, and Za (lanes 7, 8), 55 μg. C1$\bar{s}$, 7.5 μg in all mixtures. The anode is down. Even though C1-INH At was clearly cleaved into lower mol wt fragments by C1$\bar{s}$, two high mol wt complex bands stable in SDS were also formed. C$\bar{1}$-INH Bo, which failed to interact with kallikrein in the experiment in Figure 3, did form a small amount of stable complex with C1$\bar{s}$. C$\bar{1}$-INH Za and C1$\bar{s}$ formed two high mol wt bands, but the source of the two bands is not clear.

Interaction of C1$\bar{s}$ with dysfunctional C$\bar{1}$-INH. When dysfunctional C$\bar{1}$-INHs from At, Bo, and Za were examined in 7.5% polyacrylamide gels, C$\bar{1}$-INH At formed two high mol wt complexes with C1$\bar{s}$, and lower mol wt cleavage fragments evolved (Fig 4, lane 2). C$\bar{1}$-INH Bo and Za also formed high mol wt complexes with C1$\bar{s}$, whereas neither did so with kallikrein. The mol wt of the major C$\bar{1}$-INH Za complex with C1$\bar{s}$ was lower than that formed with C$\bar{1}$-INH Bo but about the same as the lighter complex between C$\bar{1}$-INH At and C1$\bar{s}$. Cleavage of dysfunctional C$\bar{1}$-INH Ta and We by C1$\bar{s}$ was observed in studies reported earlier.[8]

We have not detected cleavage fragments of less than 96,000 mol wt in SDS gels when normal C$\bar{1}$-INH has been exposed to C1$\bar{s}$. The interactions of each of these C$\bar{1}$-INH proteins with C1$\bar{s}$ and kallikrein are compared in Table 1.

DISCUSSION

When normal C$\bar{1}$-INH, which was a doublet in SDS-polyacrylamide gel electrophoresis, was incubated with kallikrein and then subjected to electrophoresis in the same gel system, a major high mol wt complex of about 185,000 to 190,000 mol wt could be visualized (Fig 1A), but only the component of C$\bar{1}$-INH having a mol wt of approximately 106,000 formed stable complexes with kallikrein. The lower mol wt band in the C$\bar{1}$-INH preparation (approximately 96,000) did not do so (Figs 1A and 2). When increasing amounts of C1$\bar{s}$ were incubated with the same preparation of C$\bar{1}$-INH before electrophoresis in graded concentrations of polyacrylamide, both the lower and higher mol wt components of C$\bar{1}$-INH formed a stable complex with C1$\bar{s}$ (Fig 1B). Therefore the 96,000 mol wt component of normal C$\bar{1}$-INH apparently contained structural requirements for stable complex formation with C1$\bar{s}$ but not with kallikrein. Both enzymes could generate the doublet C$\bar{1}$-INH from a preparation containing a homogeneous single band of protein (Fig 2). Exposure to kallikrein appeared to generate a lower mol wt cleavage fragment than that generated by C1$\bar{s}$ (Figs 1A and 1B), as if kallikrein degraded the protein further than did C1$\bar{s}$. Van der Graaf and his associates also found that a plasma kallikrein preparation, or its light chain failed to form complexes with a lower mol wt form (94,000) of C$\bar{1}$-INH,[16] which may be the same as the lower mol wt C$\bar{1}$-INH seen in the present studies. The fact that some preparations of C$\bar{1}$-INH, which are isolated by the method of Harrison,[9] are in a doublet form implies that the protein may have had contact with C1$\bar{s}$ or kallikrein in vivo, for isolation was carried out in the presence of exogenous inhibitors aimed at preventing cleavage of C$\bar{1}$-INH in vitro. One of us (Harrison), however, has found only a single band of C$\bar{1}$-INH antigen in freshly drawn normal plasma using a monoclonal antibody (MoAb) to C$\bar{1}$-INH in an electroimmunofixation procedure. Harpel and Cooper[17] identified a plasmin-generated cleavage fragment of normal C$\bar{1}$-INH that was initially functional but lost activity without evidence of a further loss in mol wt. Zuraw and Curd recently reported that an inactive fragment of 94,000 mol wt could be released from normal C$\bar{1}$-INH in plasma when kallikrein was activated.[17a]

The interactions of dysfunctional C$\bar{1}$-INH proteins with plasma kallikrein were heterogeneous, and only two of these formed complexes with kallikrein that were stable during electrophoresis in graded concentrations of polyacrylamide containing SDS. Each of these (C$\bar{1}$-INH At and Za) had reduced inhibitory properties directed against the amidolytic activity of plasma kallikrein in studies previously reported[8] (Table 1). Therefore the complexes visualized in these studies may not be associated with effective inhibition of kallikrein by the C$\bar{1}$-INH proteins. Dysfunctional C$\bar{1}$-INH We also had reduced inhibitory properties directed against C1$\bar{s}$ but formed visible complexes with C1$\bar{s}$ (Table 1). C$\bar{1}$-INH Bo was cleaved by the plasma kallikrein into multiple lower mol wt fragments of less than 90,000 mol wt (Fig 3). In earlier studies C$\bar{1}$-INH We was exceedingly susceptible to plasmin cleavage.[8] These observations reinforce the evidence of structural heterogeneity of these dysfunctional proteins. It is likely that point mutations account for many of the alterations in dysfunctional C$\bar{1}$-INH proteins,[18] but in at least one instance (C$\bar{1}$-INH Ta) there is an insertion in a high mol wt cyanogen bromide fragment[19] and in another (C1-INH We) a deletion as well as an insertion.[20]

Three dysfunctional C$\bar{1}$-INH proteins were examined for their interactions with preparations of C1$\bar{s}$, since these had not been tested earlier (Fig 4). One, C$\bar{1}$-INH At, was cleaved by C1$\bar{s}$ (Fig 4), and high mol wt complexes were seen, while two others (C$\bar{1}$-INH Bo and Za) also formed a high mol wt complex with C1$\bar{s}$ but were not cleaved when examined under the same conditions of incubation and electrophoresis. C$\bar{1}$-INH Za formed a stable complex with C1$\bar{s}$ (Fig 4) but not with kallikrein, but it inhibited C1$\bar{s}$ to a lesser degree (19% of normal) than kallikrein (64% of normal) in earlier studies.[8] In this instance, the complex between this C$\bar{1}$-INH and C1$\bar{s}$, the C1$\bar{s}$ does not appear to be inhibited; this is reminiscent of our earlier studies in which noninhibitory complexes between plasmin and some of these dysfunctional C$\bar{1}$-INH proteins (We, Da, Za, and Ri) were identified.

These studies point to the possibility that the C$\bar{1}$-INH found in normal plasma may have more than one point of interaction with plasma kallikrein, some of which are defective in dysfunctional C$\bar{1}$-INH proteins. Since most of the dysfunctional C$\bar{1}$-INH proteins did not form complexes of sufficient stability to be visible in the SDS gels but did provide measurable degrees of inhibition in work previously reported,[8] complexes were probably formed but were dissociated during the procedures used for electrophoretic examination of complexes. Some of the inhibitory activities measured may have been due to trace amounts of normal C$\bar{1}$-INH not distinguished from the dysfunctional forms in these electrophoretic analyses. It is likely that the binding affinity of most of the dysfunctional C$\bar{1}$-INH proteins for plasma kallikrein is considerably less than that of normal C$\bar{1}$-INH, and, with two exceptions, significant amounts of covalently linked complexes were apparently not formed.

Serine proteases cleave inhibitors before forming functionally inhibited complexes.[21-24] Salveson and his associates[24] recently identified cleavage of normal C$\bar{1}$-INH by a preparation of C1$\bar{s}$, comparable to that used in the present experiments, by defining the amino acid sequence of a portion of the cleavage fragment released from the carboxy-terminal side of the reactive site of C$\bar{1}$-INH. Weiss and Engel[25] reported that heparin could enhance cleavage of C$\bar{1}$-INH by C1$\bar{s}$. In our experiments a homogeneous preparation of normal C$\bar{1}$-INH (mol wt approximately 106,000) was cleaved by C1$\bar{s}$ and kallikrein, forming a doublet of which the mol wt of the lighter component was 96,000. Bing and Canar[26] have identified a 90,000 to 92,000 mol wt fragment that could be generated by plasmin. Neither C1$\bar{s}$ nor kallikrein appeared to release a similar fragment in their experiments or those reported here. It is possible that some fragments of C$\bar{1}$-INH of much lower mol wt may have been released from C$\bar{1}$-INH protein during their interactions with kallikrein but were not detected because the heavily glycosylated portions of the molecule may not bind to protein stains.

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In Vitro Action of Tumor Necrosis Factor on Myeloid Leukemia Cells

By Reinhold Munker and Phillip Koeffler

We investigated the in vitro action of recombinant tumor necrosis factor alpha (TNF) on the clonal growth of normal and malignant myeloid cells. Clonogenic cells from six of nine myeloid leukemia cell lines were very sensitive to the effects of TNF with 50% of the colonies inhibited (ED_{50}) by concentrations of TNF that ranged between 6 and 150 U/mL. A decrease in DNA, RNA, and protein synthesis and in cloning efficiency occurred within three hours of exposure of HL-60 promyelocytes to TNF. The TNF in combination with recombinant interferons produced an additive or synergistic inhibition of colony formation of HL-60 and THP-1 myelomonoblasts. Normal human CFU-GM are sensitive to TNF (ED_{50} between 100 and 50,000 U/mL), but their sensitivity to TNF depends on the source of colony stimulating factor (CSF) with T lymphocyte derived GM-CSF (recombinant or natural) partially protecting the CFU-GM from the suppression exerted by TNF (and interferons). In eight of 15 cases the clonogenic myeloid leukemia cells from patients with acute or chronic myeloid leukemia were more sensitive than normal CFU-GM using GM-CSF as a source of colony stimulating activity. Further studies showed that the action of TNF on myeloid leukemia cells probably can be only partially explained by differentiation. Our finding of a possible selective cytotoxicity to leukemic clonogenic cells by TNF suggests that TNF may have value in the therapy of some patients with myeloid leukemia.

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DESPITE recent improvements in chemotherapy and bone marrow transplantation, the prognosis of acute and chronic myelogenous leukemia is unsatisfactory.[1,2] Therefore new drugs and therapeutic possibilities continue to be investigated. Tumor necrosis factor (TNF) was discovered when mice with transplanted tumors developed hemorrhagic necrosis of these tumors when endotoxin was injected after priming with Bacillus calmette guerin (BCG). Activated macrophages were identified as the source of TNF.[3,4] Growth of a variety of mouse and human tumor cells are inhibited by TNF, whereas several types of normal cells from the same species are unaffected after exposure to the protein.[3] Recently the gene for human TNF was cloned and recombinant proteins synthesized.[5-7] We studied the effects of recombinant human TNF on human myeloid clonogenic cells from leukemia cell lines, patients with leukemia, and normal individuals to determine if the proliferation of malignant and normal myeloid progenitor cells is sensitive to this monokine.

MATERIALS AND METHODS

Details and references concerning the myeloid cell lines used in this study are provided in Table 1. The cells were grown in Lux tissue culture flasks in alpha medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum (FCS). Cells in logarithmic growth were used for plating experiments. Cell concentration and viability were determined by counting the cells at various time points in 0.4% trypan blue (Gibco Laboratories, Chagrin Falls, OH).

Bone marrow was obtained from both healthy volunteers and leukemic patients by aspiration from the iliac crest after written consent was obtained. For anticoagulation, preservative-free heparin (O'Neill, Jones and Feldman, St. Louis) was used. The mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients, washed twice in phosphate-buffered saline (PBS), and suspended in alpha medium containing 10% FCS and 1% penicillin-streptomycin (Irvine Scientific, Irvine, CA). Prior to use in plating experiments the light density bone marrow cells from normal volunteers were incubated for 90 minutes on plastic surfaces in a humidified, 5% CO_2, 37°C incubator to deplete adherent cells. Peripheral blood mononuclear cells were obtained from leukemia patients using similar techniques. Details concerning the leukemia patients are provided in Table 2. Bone marrow and peripheral blood samples contained more than 90% leukemic cells as determined by morphology. Leukemia cells from all patients with chronic myelogenous leukemia contained the Philadelphia chromosome.

Reagents. Recombinant human tumor necrosis factor[5] was generously provided by Genentech, San Francisco, and was stored at 4°C (1 mg corresponds to 3.7×10^7 U). Before use, dilutions in tissue culture medium containing 10% FCS were made. Recombinant interferons were kindly provided as follows: alpha interferon (1.8×10^7 U/mL) by Hoffmann-La Roche, Nutley, NJ; beta interferon (1 mg = 45×10^6 U) by Cetus, Emeryville, CA; gamma interferon (2 to 3×10^7 U/mg) by Genentech, San Francisco.

Colony formation in soft agar. Cells were plated in Lux culture dishes (Miles Lab, Naperville, IL) in a two-layer soft agar system according to previously described methods.[17] The underlayer contained 0.5% agar, the upper layer 0.3% agar (Difco, Detroit). The culture medium was alpha medium. The TNF and, if appropriate, CSF were mixed into the lower layer. Several sources of CSF were used: conditioned medium from a human T lymphocyte line (MO)[18]; human placenta conditioned medium (CM) day 6 [prepared as described by Burgess et al[19]; recombinant human GM-CSF[20] was provided by Dr J.C. Gasson; GCT-conditioned medium[21] was kindly provided by Drs J. Di Persio (UCLA) and C. Abboud (University of Rochester); Dr L. Souza (Amgen, Thousand Oaks, CA) generously provided recombinant G-CSF.[22] All sources of CSF were tested at different concentrations and used at the optimal dose: 5% CM for placental and GCT-conditioned medium, 4% to 5% CM for Mo; 100 pM for recombinant GM-CSF; 25 ng/mL for recombinant G-CSF. The FCS used was prescreened for low background colony formation

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Table 1. Effects of Tumor Necrosis Factor on Clonal Growth of Human Myeloid Leukemia Cell Lines

Cell Line	Reference	Description	ED$_{50}$* (U/mL)
HL-60	8	Promyelocytes	6
HL-60 variant	9	Undifferentiated blasts	7
EM-3	10	Myeloblasts	8
U937	11	Monoblasts	12
KG-1	12	Myeloblasts	45
KG-1A	13	Early myeloblasts	150
HEL	14	Bipotent‡	†
K562	15	Bipotent‡	†
THP-1	16	Monoblasts	†

*50 percent inhibitory concentration of TNF

†Less than 50 percent inhibition at 10,000 U/ml TNF

‡Monoblast and erythroblast characteristics

and high stimulation with CSF. For normal human bone marrow and leukemic cells, 2 × 10^5 cells were plated per dish. Myeloid cell lines were plated at 1,000 to 5,000 cells per dish. The plating efficiency varied between 5% (KG-1) and 38% (K562). The KG-1 cell line (passage 28) formed colonies only in the presence of CSF. Cultures were placed in a humidified atmosphere, 5% CO$_2$, 37°C. Normal CFU-GM (colonies of ≥40 cells) and leukemic colonies (aggregates of ≥20 cells) were scored after ten days with an inverted microscope. All experiments were performed using triplicate or quadruplicate plates per experimental point.

In some experiments HL-60 cells were incubated with different concentrations of TNF (20, 50, 500, and 5,000 U/mL) for various time points and were washed extensively. The number of surviving cells was determined by counting in trypan blue solution. At each time point 2,000 viable cells/plate were seeded. Depending on the concentration of TNF (5,000 to 20 U/mL), 12% to 97% of the cells survived a 24-hour incubation compared to control cultures. To demonstrate the specificity of the effects of TNF, 20,000 U were incubated at room temperature with 20 μg of neutralizing monoclonal antibody (MoAb) against TNF (kindly provided by Dr C. Nelson, Genentech, San Francisco) for 90 minutes and then used in plating experiments as described.

Table 2. Effects of Tumor Necrosis Factor on Clonal Growth of Myeloid Leukemia Cells From Patients

Patient No.	Initials	Age (years)	Diagnosis	Cell Source	Colonies*	ED$_{50}$† (U/mL)
1	BE	63	AML	PB	183	450
2	MU	18	AML	BM	154	‡
3	CR	41	AML	BM	84	250
4	SE	77	AML	BM	74	20
5	HD	20	AML	BM	114	2,500
6	SL	27	CML	PB	22	150
7	LA	36	CML	PB	42	500
8	HD	27	CML	PB	32	80
9	BB	34	CML	PB	28	1,000
10	DG	26	CML	PB	219	‡
11	SF	64	CML	PB	63	250
12	BL	63	CMLmyBC	BM	97	50
13	NR	47	CMLmyBC	PB	165	20
14	KD	25	CMLmyBC	PB	95	‡
15	MM	30	CMLmyBC	BM	252	250

Abbreviations: AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; myBC, myeloid blast crisis; PB, peripheral blood; BM, bone marrow.

*Number of colonies per 2 × 10^5 cells plated in dishes not containing TNF.

†50% inhibitory concentration of TNF.

‡Less than 50% inhibition at 10,000 U/mL of TNF.

DNA, RNA, and protein synthesis. The DNA synthesis was studied by exposing TNF-treated cells (10^6/mL) to 0.5 μCi methyl ^{3}H-thymidine (NEN, Boston) for 90 minutes in multiwell tissue culture plates (quadruplicate wells per point) in a humidified atmosphere with 5% CO$_2$. The cells were harvested with a MASH-harvester, and isotope uptake was determined with liquid scintillation photometry (Beckman [S1800]). The RNA synthesis was studied by labeling TNF-treated cells (10^6/mL) with 0.5 μCi ^{14}C-uridine (UdR) for two hours at 37°C (triplicate wells per point), washing the cells twice in PBS, precipitating the cells on ice with 5% trichloroacetic acid (TCA) for ten minutes, washing twice with 5% TCA, and heating for 60 minutes at 80°C. A 200 μL aliquot from each sample was mixed with Aquasol (NEN, Boston) and counted in a scintillation photometer. Protein synthesis was determined by suspending the cells (75,000 cells per microtiter well) in methionine-free Earle's medium (Gibco, Grand Island, NY) with 5% FCS and exposing the cells to 1 μCi ^{35}S-methionine for 90 minutes. Protein synthesis was determined as described for DNA synthesis. The results for DNA, RNA, and protein synthesis were corrected for cell loss during incubation with TNF.

NBT reduction, nonspecific esterase cytochemistry. For NBT reduction, cells were mixed with an equal volume of a solution containing 1.25 mg/mL NBT (Sigma, St. Louis), 17 mg/mL bovine serum albumin and 1 μg/mL 12-0-tetradecanoylphorbol 13-acetate (Miles) for 30 minutes in 37°C incubator with 5% CO$_2$; washed in PBS, cytocentrifuged; fixed in methanol; and stained with Giemsa solution. For cytochemistry the cells were stained for alpha-naphthyl-acetate esterase (ANAE), according to routine hematologic methods (kindly performed by Dr F. Naiem, UCLA Dept of Pathology).

Statistical comparisons. We define synergism to mean the proportion of colony growth in the presence of TNF and IFN < (proportion of colony growth in the presence of TNF alone) × (proportion of colony growth in the presence of IFN alone). Therefore synergism may be evaluated by comparing the logarithm of colony growth in the presence of TNF and IFN to the sum of the logarithms of growth in TNF alone and IFN alone. For statistical analysis we made graphic comparisons and used Student's *t* test to detect synergism at combinations of TNF and interferons and for the comparison of different points (analysis kindly performed by Dr D. Heitjan, UCLA Dept of Biomathematics).

RESULTS

Clonal proliferation of cells from six of nine myeloid leukemia cell lines was inhibited by recombinant TNF (Fig 1, Table 1, mean values of at least three independent experiments). The concentration required for a 50% inhibition of colony formation in soft agar (ED$_{50}$) varied between 6 and 150 U/mL. Colony formation of sensitive lines was inhibited almost 100% at 1,000 U/mL. Cell lines that did not reach a 50% inhibition at 10,000 U/mL TNF nevertheless were still clearly inhibited at this concentration. The effects observed are due to TNF since a preincubation with a neutralizing MoAb could prevent 97% ± 2% (±SD) of the inhibition of HL-60 cells (data not shown). In further experiments we exposed HL-60 to TNF for different periods of time, washed thoroughly the cells three times, counted viable cells, and plated them in soft agar. The cloning efficiency was drastically reduced after a short exposure to TNF (Fig 2). For example, after a three-hour exposure

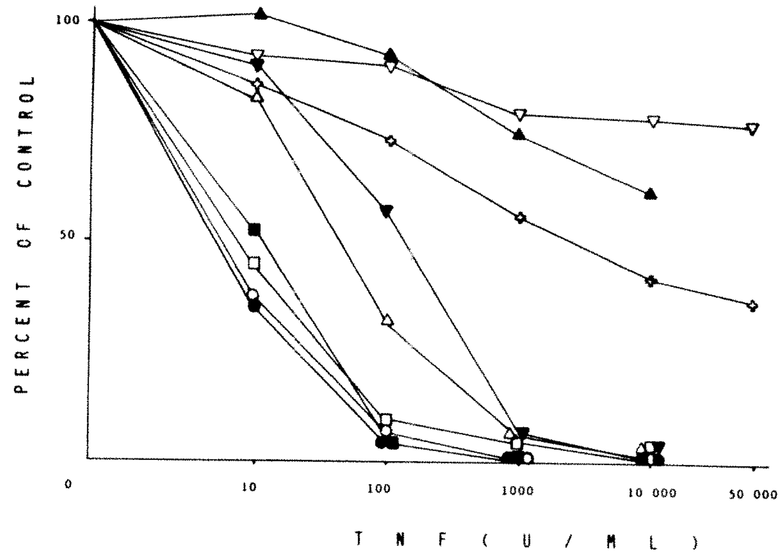

Fig 1. Effect of tumor necrosis factor on clonal growth of cells from myeloid lines. HL-60 (●), HL-60 variant (O), KG-1 (△), KG-1A (▼), EM-3 (□), U937 (■), HEL (✣), THP1 (▽), K562 (▲). Results are expressed as a percent of colonies in control dishes not containing TNF; each point represents the mean of three to seven experiments with triplicate plates per experiment.

(37°C) to 50 U/mL TNF, greater than 75% of colonies from HL-60 were inhibited. The lowest concentrations of TNF (20 U/mL) caused a less pronounced decrease in cloning efficiency than the other concentrations tested. After 24 hours a partial recovery in the cloning efficiency was observed, which may be due to lysis of the colony-forming cells most sensitive to TNF. Similar results were observed with pulse exposure of the cells to TNF at either 23°C or 4°C (data not shown).

We tested the potency of the combination of recombinant interferon and recombinant TNF on clonal inhibition of leukemic cell lines. A cell line that was relatively insensitive to TNF (THP1 myelomonoblasts) was cultured in the presence of TNF (0 to 50,000 U/mL) and either α, β, or γ recombinant interferons (0 to 5,000 U/mL). A synergistic interaction between TNF ($\geq$100 U/mL) and interferons could be observed at concentrations of either α or β interferon at $\geq$500 U/mL, and γ interferon at $\geq$1,000 U/mL. Figure 3A shows the results for the combination experiments with 5,000 U/mL of either α, β, or γ interferon and 0 to 1,000 U/mL TNF using THP1 as test cells. In contrast, TNF and γ interferon separately cultured with HL-60 in soft agar markedly inhibited clonal growth, but combinations of TNF and γ interferon produced only an additive inhibition of clonal growth of HL-60 (Fig 3B). The clonal growth of another cell line (HEL) was moderately inhibited by TNF but not by interferons; combinations of these agents neither caused additive nor synergistic inhibition of clonal growth of HEL cells (data not shown).

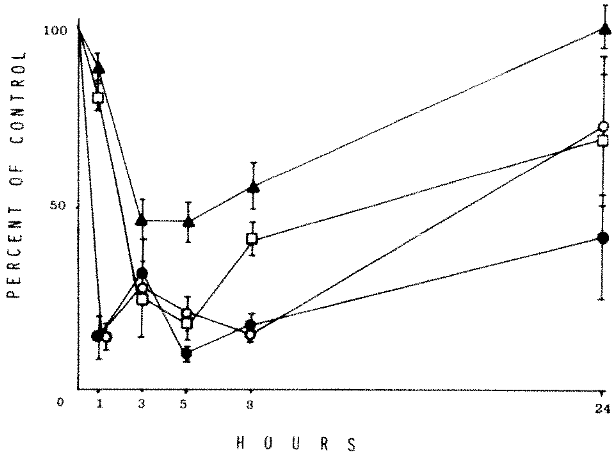

Fig 2. Pulse exposure of HL-60 cells to tumor necrosis factor. The cells (10^6/mL) were exposed to 20 (▲), 50 (□), 500 (O), 5,000 (●) U/mL TNF for the indicated time, washed, and 2,000 viable cells per point were plated in quadruplicate. Colonies were counted after ten days. Each point represents the mean ± SD of two experiments.

Low doses of TNF (20 U/mL for six days) induced a partial differentiation of the HL-60 line with 25% of cells developing the ability to reduce NBT and 8% of cells staining positively with ANAE, an enzyme that is found in monocytes and macrophages. The viability of the cells on day 6 of culture was $\geq$90%. The TNF was unable to induce differentiation of KG-1 myeloblasts or HL-60 variant blast cells.

Fresh leukemia cells from 12 of 15 patients with myeloid leukemia were sensitive to TNF in the clonogenic assay for leukemic cells, with an ED_{50} in the range of 20 to 2,500 U/mL (Table 2, Fig 4). The cells of four of five cases of AML, five of six cases of CML in chronic phase, and three of four cases of CML in myeloid blast crisis responded to TNF with a clonal inhibition. No obvious correlation between the in vitro response to TNF and the induction of remission or survival could be observed in the 13 patients in whom follow-up was available. We tested CFU-GM from nine normal donors and found that TNF also suppressed normal myeloid progenitors (Fig 5). However, the degree of suppression strongly depended on the source of colony stimulating factor used in the assay. In plates containing 10,000 U TNF/mL, a suppression of 53% was observed with MoCM (CM from HTLV-II-infected T lymphocyte line), 48% with recombinant GM-CSF, 97% with placental-conditioned medium, 98% with GCT-CM (CM from sarcoma line), and 100% with recombinant G-CSF. The 50% inhibitory concentration (ED_{50}) varied between 100 U/mL (recombinant G-CSF) and 50,000 U/mL (recombinant GM-CSF). (See

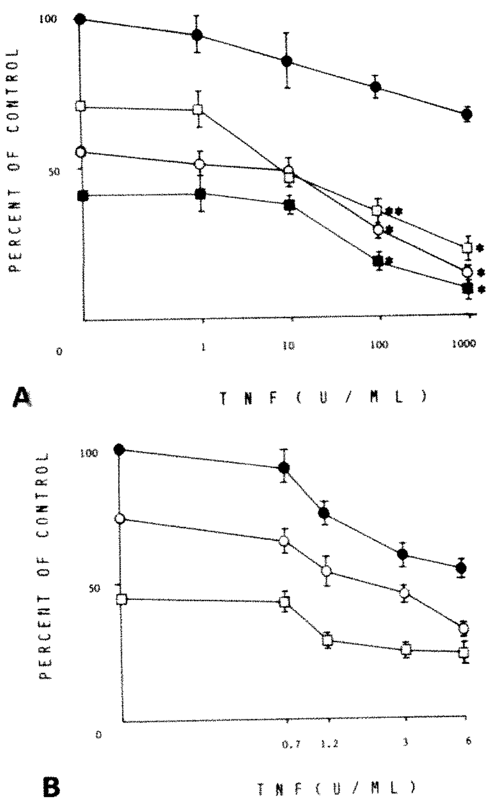

Fig 3. Effect of tumor necrosis factor and interferon on leukemic colony formation. (A) The THP1 myelomonoblast line was grown in soft agar with different concentrations of TNF alone (●) or in combination with recombinant interferons (5,000 U/mL alpha interferon [○], 5,000 U/mL beta interferon [■], 5,000 U/mL gamma interferon [□]). Synergistic inhibition is shown by one ($P \leq 0.01\%$) or two ($P \leq 0.05\%$) asterisks. The effects of IFN alone are provided at the 0 concentration. (B) The HL-60 promyelocyte line was grown in soft agar with TNF alone (●) or in combination with recombinant gamma interferon (8 U/mL [○], 24 U/mL [□]). Results for 3A and 3B are expressed as a mean percent of colonies formed in experimental plates as compared to control dishes not containing TNF or interferon; each point represents the mean ± SD of four experiments performed with triplicate plates. The effects of interferon alone are shown at the 0 concentrations of TNF.

Fig 5 for details of the inhibition curves). The TNF dose-response curves of normal human GM-CSF stimulated with conditioned medium from a HTLV-1-infected T lymphocyte line (SLB-1)[23] as a source of CSF was similar to recombinant GM-CSF and MoCM (data not shown).

We also studied the effect of combinations of recombinant α, β or γ interferons with TNF on normal committed myeloid progenitors (Table 3). In the presence of T cell-derived GM-CSF, little inhibition was observed with interferons alone or in combinations with TNF. Using either placental conditioned medium or recombinant G-CSF as sources of colony stimulating activity, the interferons were clearly inhibitory, and the addition of TNF produced an additive or synergistic effect (especially at 100 U TNF/mL).

We examined DNA, RNA, and protein synthesis in HL-60 cells in liquid culture after exposure to TNF (20 to 500 U/mL) (Fig 6). A dose-dependent decrease in all parameters was observed with increasing concentrations of TNF, with a significant ($P < 0.05$) decrease in all three parameters occurring within two hours of exposure of the cells to TNF.

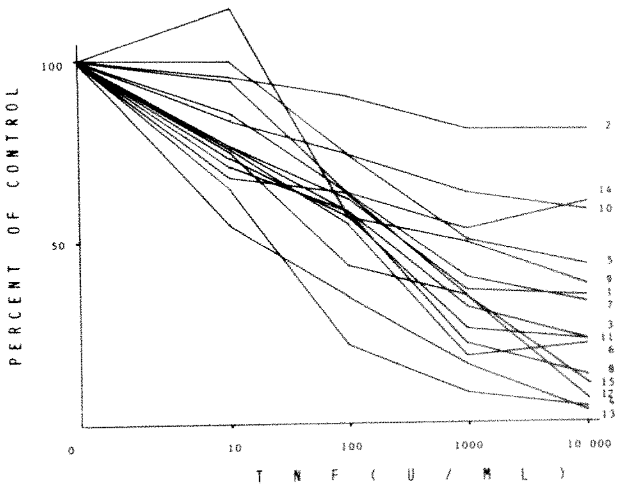

Fig 4. Effect of tumor necrosis factor on clonal growth of myeloid leukemic cells from patients. Results are expressed as a mean percent of colonies formed in experimental plates as compared to control dishes not containing TNF. Each point represents the mean of triplicate plates. Numbers on the right correspond to the patient numbers in Table 2.

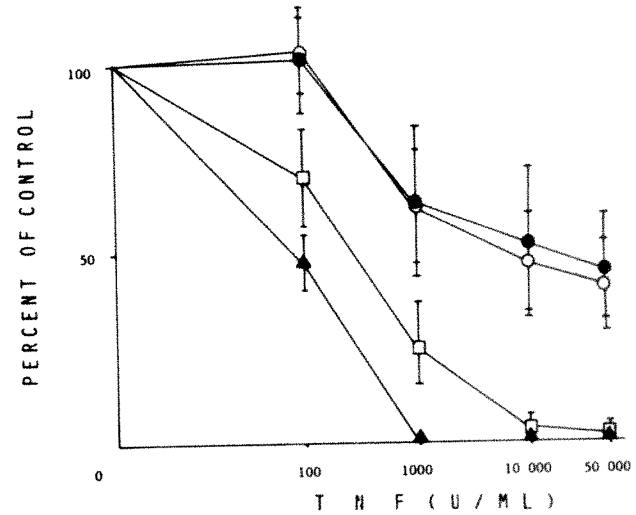

Fig 5. Effect of tumor necrosis factor on the clonal growth of CFU-GM from bone marrow of normal donors. 2 × 10^5 light density bone marrow cells (depleted of adherent cells) were plated in soft agar with various concentrations of TNF and different sources of colony stimulating factors (-0-Mo CM [5%], n = 9 donors, 153 ± 27 colonies/control plates containing no TNF; recombinant GM-CSF [●], 100 PM, n = 7 donors, 162 ± 29 colonies/control plates; placental-conditioned medium [□], 5%, n = 5 donors, 155 ± 30 colonies/control plates; recombinant G-CSF, 25 ng/mL [▲], n = 4 donors, 170 ± 15 colonies/control plates). Results are expressed as a mean percent (± SD) of colonies formed in experimental plates as compared to control dishes not containing TNF. Each experiment was performed with quadruplicate plates per point.

Table 3. Effects of Interferons, TNF, and Combinations of TNF and Interferons on Normal Myeloid Progenitors (CFU-GM)

Source of Colony Stimulating Factor	Concentration and Type of Interferon	Colony Formation (% of Control Plates without TNF or Interferons) Concentration of TNF 0	10	100 U/mL
MoCM	0 Interferon	100%	96% ± 7%	98% ± 5%
	1000 U/mL α IFN	88% ± 10%	92% ± 6%	90% ± 5%
	2000 U/mL β IFN	103% ± 5%	96% ± 12%	91% ± 9%
	1000 U/mL γ IFN	93% ± 11%	95% ± 8%	90% ± 12%
PCM	0 Interferon	100%	92% ± 10%	71% ± 15%
	1000 U/mL α IFN	70% ± 15%	53% ± 4%	20% ± 8%*
	1000 U/mL β IFN	83% ± 4%	77% ± 12%	34% ± 18%
	1000 U/mL γ IFN	69% ± 11%	51% ± 8%	13% ± 4%*
Recombinant G-CSF	0 Interferon	100%	74% ± 16%	45% ± 8%
	1000 U/mL α IFN	49% ± 10%	38% ± 5%	7% ± 6%*
	1000 U/mL β IFN	60% ± 12%	41% ± 15%	8% ± 5%*
	1000 U/mL γ IFN	33% ± 2%	23% ± 5%	3% ± 2%*

Soft agar cultures were performed as described in "Materials and Methods." Results are mean (±SD) of four experiments and are expressed as percent of control cultures without TNF or interferon. In control cultures with MoCM 149 ± 23 colonies, in control cultures with PCM 158 ± 30 colonies, and in control cultures with recombinant G-CSF 170 ± 15 colonies per plate were observed.

Abbreviations: CM, conditioned medium; PCM, placental conditioned medium, recombinant G-CSF.

*Synergistic inhibition ($P \leq 0.05\%$).

DISCUSSION

Our results suggest that TNF is inhibitory in vitro against most myeloid leukemia clonogenic cells from established lines and from patients. Recombinant TNF was recently shown to have an antiproliferative effect on seven of 22 nonhematopoietic human tumor lines, whereas five normal human diploid lines were enhanced in their growth when cultured with TNF.[24] We confirm here studies by others that TNF is inhibitory for normal CFU-GM.[25,26] Differences between investigators in the concentrations of TNF that suppress proliferation are at least partially due to how the unitage of TNF was originally determined (eg, in the presence or absence of Actinomycin D). We observed a synergistic inhibition of CFU-GM when α- and γ-interferons were combined with TNF in the presence of placental CM and recombinant G-CSF (β interferon was synergistic only in combination with G-CSF). Earlier studies showed that interferons are potent inhibitors of myeloid progenitors.[27,28] Furthermore, Broxmeyer et al[26] found synergism between TNF and γ-interferons, but not between TNF and α-interferons using normal marrow target cells. Why the results of studies by Broxmeyer et al[26] and us differ when examining the combined effects of α-interferon and TNF might relate to differences in concentrations of α-interferon or in the source of CSF used in the two studies. A new finding of this investigation is that the source of CSF determines the degree of suppression by TNF. A strong suppression of myeloid colonies by TNF occurs in the presence of recombinant G-CSF or conditioned from placenta and the GCT cell line; T lymphocyte derived GM-CSF (recombinant and natural) partially protects the myeloid progenitors from the inhibitory effects of TNF. We also observed a similar protection of myeloid colony formation by T lymphocyte derived GM-CSF against the suppressive influences of interferons.

Further studies are required to determine the mechanism

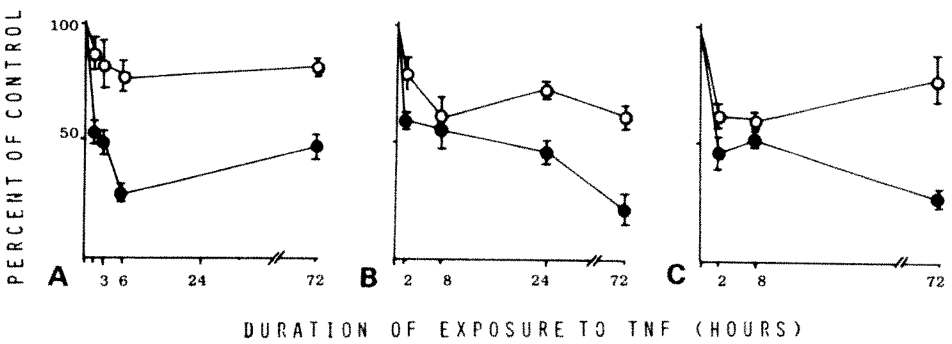

Fig 6. Influence of tumor necrosis factor on DNA, RNA, and protein synthesis of HL-60 cells. The HL-60 cells were incubated with TNF (25 [○] and 500 [●] U/mL for DNA synthesis and 20 [○] and 300 [●] U/mL for RNA and protein synthesis), for the indicated time periods, washed, and labeled with radioactive precursors. Results are expressed as a mean percent value in experimental dishes as compared to control dishes containing no TNF. Each point represents the mean of three experiments performed with quadruplicate cultures. (A), DNA-synthesis; (B), RNA-synthesis; (C), protein synthesis.

by which T lymphocyte derived GM-CSF partially reverses the inhibitory action of TNF; (eg, a direct interaction with the TNF protein, competition for the TNF receptor, stimulation of a clonogenic myeloid progenitor cell that is partially resistant to TNF, stabilization of protein kinase C).

The mechanism of inhibition of proliferation of clonogenic leukemia cells mediated by TNF is unclear but probably is not completely linked to differentiation, since several of the most sensitive myeloid lines (KG-1, HL-60 blasts) were not induced to differentiate when exposed to TNF. Approximately 25% of HL-60 cells differentiated after a six-day exposure to TNF (20 U/mL). Takeda et al[29] have provided evidence that TNFα is the same as a previously reported protein known as differentiation inducing factor, which can induce differentiation of HL-60 cells. Recently receptors for TNF were demonstrated on the surface of several cell types.[24,30,31] However, the presence of TNF receptors on the cell does not necessarily predict that a cell will be sensitive to TNF, since a TNF resistant cell line has been reported with approximately the same number of TNF receptors as a cell line sensitive to the cytotoxic effects of TNF.[24] The synergism of TNF with gamma interferon, which was originally observed by Williamson et al[31] and which we found for THP1 myelomonoblast cells, may be due to an increase of the number of TNF receptors as has been reported for a cervical carcinoma cell line.[32] We also observed that the cloning efficiency and the DNA, RNA, and protein synthesis of HL-60 cells decreased within two hours of exposure to TNF, suggesting that TNF very rapidly affects vital cellular functions.

When we compared the inhibition of leukemic clonogenic cells with the inhibition of normal CFU-GM using T cell derived GM-CSF, in eight of the 15 cases (two AML, three CML in the chronic phase, and three cases in blast transformation), the leukemic cells were more sensitive than the mean ± SD of normal CFU-GM.

The reason why most malignant myeloid cell populations are sensitive to the growth inhibiting action of TNF and why normal myeloid cells may be less sensitive is unclear. Possibilities include differences in affinity or number of TNF receptors, differences in the ability to translocate TNF across the cell membrane, alteration in critical cellular substrates needed for the action of TNF, or ineffective repair mechanisms in the neoplastic cells. Our in vitro results should be interpreted with caution, since we did not prove that all in vitro cell aggregates in the soft agar cultures were derived from the malignant clone and since the in vitro clonogenic cells may not be true tumor stem cells in vivo.[24] Nevertheless TNF may possibly have value in the therapy of patients with myeloid leukemia. In vitro studies with leukemic clonogenic cells may help to identify these patients. The potential toxicity of TNF, including myelotoxicity, must be carefully evaluated, since TNF or a closely related molecule was found to mediate a variety of adverse effects in mice.[33,34]

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Fetal Hemoglobin-Containing Cells Have the Same Mean Corpuscular Hemoglobin as Cells Without Fetal Hemoglobin: A Reciprocal Relationship Between Gamma- and Beta-Globin Gene Expression in Normal Subjects and in Those With High Fetal Hemoglobin Production

By G.J. Dover and S.H. Boyer

We have developed methodology that allows comparison of the mean corpuscular hemoglobin (MCH) of fetal hemoglobin (HbF)-containing red cells (F cells) with the MCH of non-F cells from the same individual. To do this, suspensions of peripheral blood erythrocytes and their internal contents are fixed with an imidodiester, dimethyl-3,3'-dithiobispropionimidate dihydrochloride (DTBP). Thereafter fixed cells are made permeable to antisera by treatment with Triton X-100 and isopropanol, reacted with a mouse monoclonal antibody (MoAb) against HbF, and then with fluorescein-conjugated antimouse IgG. No appreciable hemoglobin is lost during such manipulation. Red cells from a diversity of subjects were thus treated and examined microscopically, first by transmitted light and then by epifluorescence. A direct correlation between Coulter-derived MCH and mean absorbance of 415 nm transmitted light was found for 100 unfixed ($r = 0.96$) and for 100 antibody-treated fixed-permeabilized red cells ($r = 0.99$) among individuals selected so as to provide a range of Coulter MCH values between 20 and 35. Comparisons of microscopically derived MCH of F cells and non-F cells were statistically nondistinguishable ($P > 0.05$) in all subjects. Such comparisons included normal individuals (less than 1% F cells), SS patients (7% to 48% F cells), subjects with congenital anemia (22% to 65% F cells), individuals with heterocellular hereditary persistence of HbF (HPFH) (12% to 21% F cells), and heterozygotes for beta$^+$ thalassemia (11% to 31% F cells). We conclude that gamma- and beta-globin production within F cells is regulated in a reciprocal fashion both among normal individuals and among individuals with elevated HbF production.

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THROUGHOUT normal adult erythropoiesis, hemolysate fetal hemoglobin (HbF) persists at a low level of usually less than 2.0%. This small amount of HbF is restricted to a subpopulation of erythrocytes, termed F cells.[1] F cells contain both HbF and adult hemoglobin (HbA). While the amount of HbF within F cells has been directly measured and found to range from 3 to 8 pg HbF/F cell,[2] it is not known whether the mean corpuscular hemoglobin (MCH) of F cells is greater than that of non-F cells from the same individual. Hence it is not clear whether F cells are (1) formed as a result of HbF being superimposed upon HbA content with the result that the MCH of F cells would become greater than that of non-F cells, or (2) the MCH is held constant among F cells and non-F cells in any one individual. The latter observation, if true, would suggest that HbF and HbA content is reciprocally regulated within F cells. In this report we introduce new methodology to compare the MCH of F cells and non-F cells. We find that F cells and non-F cells have equal MCH, both in normal adults and in individuals with increased HbF production.

MATERIALS AND METHODS

Subjects. EDTA anticoagulated blood was obtained from normal persons, from patients with sickle cell (SS) disease, and from individuals with hereditary persistence of HbF (HPFH), beta$^+$ thalassemia, or congenital anemia (Fanconi's anemia and Diamond-Blackfan anemia).

Red cell fixation. Prior to fixation, 0.5 mL whole blood was washed once with 3 mL phosphate-buffered saline PBS (20 mmol/L sodium phosphate, pH 7.4, 0.13 mol/L NaCl) and twice with 3 mL 0.125 mol/L borate-saline, pH 8.37.[3] To cross-link erythrocyte proteins and thus fix cells,[4,5] 0.1 mL of ice-cold borate saline containing 50 mg/mL dimethyl-3,3'-dithiobispropionimidate dihydrochloride (DTBP) (Pierce Chemicals, Rockford, IL) was added to 1 mL washed cell suspension having an approximately 3% hematocrit and the mixture gently rotated in a capped 1.5 mL polypropylene Eppendorf tube (Brinkman Instruments, Westbury, NY) for 30 minutes at 37°C. Thereafter unreacted DTBP was consumed by introducing the mixture into a 12 × 75-mm glass tube containing 2 mL ice-cold 62 mmol/L diethanolamine in borate saline. After brief mixing cells were pelleted by 30-second centrifugation at 1,000 × g and resuspended in 4 mL of the 62 mmol/L diethanolamine solution. Five minutes later fixed cells were recovered by centrifugation and resuspended in 2 mL PBS containing 3% bovine serum albumin and 0.025% NaN_3. To preclude later cell clumping, 1-mL aliquots of the resuspension were briefly vortexed just before storage at −85°C.

Red cell permeabilization. Ten microliters of a thawed suspension of fixed cells, containing approximately 10^6 erythrocytes, were introduced into a 1.5 mL polypropylene Eppendorf tube filled with a Millipore-filtered (Bedford, MA) solution of scintillation-grade 0.8% Triton X-100 (Research Products International, Elk Grove, IL) in PBS. After two to five minutes, cells were centrifuged for 12 seconds in a model 5413 Eppendorf centrifuge. Following careful removal of supernatant, 0.5-mL filtered 2.5% Triton X-100 in PBS was added and cells quickly resuspended by 10- to 15-second vortex mixing. So as to permeabilize cells further, 0.5 mL isopropanol was immediately added, tube contents were again vortex mixed for a few seconds, and the suspension was at once centrifuged for 30 seconds at 1,000 × g in a Clay-Adams Sero-Fuge II (Parsippany, NJ). The desired result was to deposit erythrocytes as an even band along the side of the tube, thereby avoiding unwanted clumping. After supernatant removal, cells were vortex mixed with 1 mL filtered 0.8% Triton X-100 in PBS and recovered by 12-second centrifugation in

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the Eppendorf centrifuge. Thereafter cells were resuspended in a residual supernatant volume of about 20 μL. If fixed permeabilized cells had been seen to clump during centrifugation in the Clay-Adams device, or if they were to be examined by flow cytometry, they were briefly sonicated using a cup sonicator (Heat Systems-Ultrasonics, Plainview, NY) set at maximum output until any clumps had visibly dispersed.

Monoclonal anti-HbF and immunofluorescent staining of cells. Monoclonal antibodies (MoAbs) against HbF, purified from an individual with homozygous pancellular HPFH,[6] were developed from a Balb/c mouse given (1) 100 μg of a 3:1 (wt/wt) HbA:HbA$_2$ mixture on the day of birth, in an attempt to induce tolerance to these antigens; and (2) beginning at age 4 weeks, seven intraperitoneal injections of 0.4 to 0.75 mg HbF plus Freunds adjuvant over a 10-month period. Antisera obtained just before sacrifice reacted with HbA, albeit less well than with HbF. Two days after the last injection of HbF, polyethylene glycol 4,000 (Merck, Dormstadt, Germany) was used to fuse 10^8 spleen cells from the immunized mouse with 10^8 SP2/0-Ag14 mouse myeloma cells.[7] Two weeks after seeding in the presence of peritoneal macrophages and after beginning selection with hypoxanthine-aminopterin-thymidine,[8,9] hybridomas were found in 48% of 960 culture wells. Media from the 50 wells with the most rapidly propagating cells were screened for reactivity with CO-HbF and CO-HbA affixed to Immulon (Dynatech Laboratories, Alexandria, VA) surfaces of 96-well plates. Eleven anti-HbF clones were detected following reaction with alkaline phosphatase-coupled anti-antibody. Ten of these clones reacted better with HbF than with HbA. Ascites fluids from pristane-primed mice[8,9] injected with semisoft agarose-derived subclones from one of these hybridomas, termed 15-3.1, persistently reacted at least 100-fold better with HbF than with equivalent quantities of HbA. 15-3.1 is a sturdy cell line that grows readily in culture, remains reproducible in its anti-HbF production, and has resisted loss during repeated episodes of freezing-thawing over a 5-year period. However, the affinity of the anit-HbF thus produced is comparatively low, and, while it binds readily to excesses of immobilized HbF, it reacts poorly with trace levels of HbF in solution and therefore cannot be utilized in conventional radioimmunoassays.

For F-cell identification, 20 μL of a suspension of fixed and permeabilized cells was reacted with 50 μL of 15-3.1 in the form of mouse ascites fluid containing 25 mg/mL protein and about 5 mg/mL anti-HbF. After 15-minute incubation at room temperature with periodic agitation, cells were twice washed with 1 mL filtered 0.8% Triton X-100 in PBS using an Eppendorf centrifuge. Recovered cells were then reacted with 50 μL of 0.8 mg/mL Fluorescein-conjugated F(ab')$_2$ fragment of affinity-purified rabbit antimouse IgG (Cooper Biomedical, Westchester, PA). After a 15-minute reaction in the dark, cells were twice washed with 1 mL filtered 0.8% Triton X-100 in PBS. For microscopy, cells were suspended in a solution composed of 1 vol 10 mg/mL p-phenylenediamine (Sigma, St. Louis) in PBS and 9 vol glycerol[10] and mounted on glass microscope slides under cover slips sealed with fingernail polish.

Determination of absorbance of 415 nm light by individual F cells and non-F cells. A Zeiss universal microscope equipped for both transmitted light and epifluorescence, a rapid shutter mechanism, and a photomultiplier interfaced to a Hewlett Packard 9825A computer was used for all microscopic measurements. Light from a 100 W mercury lamp was transmitted through a 415 ± 9 nm band pass filter (Infrared Industries, Waltham, MA), and the light absorbance for 100 individual red cells was measured at 500X using a Zeiss 25X Neofluar immersion objective. For each cell the light absorbance was measured in a circular field 10 μm in diameter. Background light absorbance from an adjacent field, free of cells, was subtracted from each measurement. Paired observations for the cell and background were stored and collated in the computer. Background light was kept uniform between slides by manipulation of the microscope condenser and normalization of photomultiplier output from an empty field, thereby ensuring reproducible measurements. In assays where comparisons of 415 nm light absorption from F cells and non-F cells were to be made, each cell was also viewed under >520 nm emitted epifluorescence so as to distinguish between F cells and non-F cells.

The percentage of F cells was separately determined for each sample by estimating the proportion of fluorescent cells found among 500 fixed and permeabilized erythrocytes. In addition, suspensions of fluorescent-labeled cells were analyzed in an Ortho Cytofluorograf (Ortho Instruments, Boston) equipped with 0.8 mW helium-neon and 100 mW argon lasers. Percent HbF in whole blood hemolysates was measured by alkali denaturation.[11] The number of alpha-globin genes in subjects with SS disease and microcytosis was determined via restriction enzyme analysis of DNA derived from peripheral blood leukocytes.[12] Statistical analysis of the difference between MCH of F cells and non-F cells was performed by testing the null hypothesis with Student's *t* test.

RESULTS

HbF detection with monoclonal anti-HbF. For a variety of subjects, measurements of the percentage of F cells, as determined by visual enumeration of fluorescent cells labeled with monoclonal anti-HbF (15-3.1) and antimouse IgG, were no different (data not shown) than the percentage of F cells detected by immunoprecipitate assays with polyclonal rabbit anti-HbF antibodies.[13] The same was true when suspensions of permeabilized, fixed red cells, reacted with the same monoclonal anti-HbF and secondary antibody, were assayed by flow cytometry. As seen in Fig 1, two populations of cells with distinctive fluorescent levels were evident in flow cytometry assays. For each subject illustrated in Fig 1, the percentage of all red cells that exhibited the higher fluorescence level was indistinguishable from the percentage of F cells identified visually at the microscope.

MCH v 415 nm light absorbance of individual erythrocytes. The mean absorbance of 415 nm light for 100 untreated RBCs was identical to that of 100 fixed, permeabilized RBCs. For example, in one subject, average light absorption (±SD) for fresh RBCs was 110 ± 28 absorbance units, while that of fixed and permeabilized RBCs was 111 ± 28 units. Additions of MoAb and fluorescent secondary antibody did not affect these mean values. Such measurements verified that no hemoglobin is lost during the fixation and permeabilization procedures. Furthermore, cell morphology is maintained. For example, biconcave discs, spherocytes, and reversible and irreversible sickle forms were readily identifiable in permeabilized, fixed cells in the same proportion as in untreated cells.

To transform absorbance of 415 nm light into MCH, mean absorbance for 100 RBCs from each of a series of individuals was plotted against whole blood MCH measured in a Coulter Model S (Coulter Instruments, Hialeah, FL). As apparent in Fig 2A, there was a close correlation (r = 0.96) between mean 415 nm light absorbance by fresh RBCs suspended on glass slides and the MCH as determined by a Coulter counter. Figure 2B demonstrates that similar measurements performed on DTBP-fixed permeabilized RBCs, to which monoclonal anti-HbF and fluorescent anti-

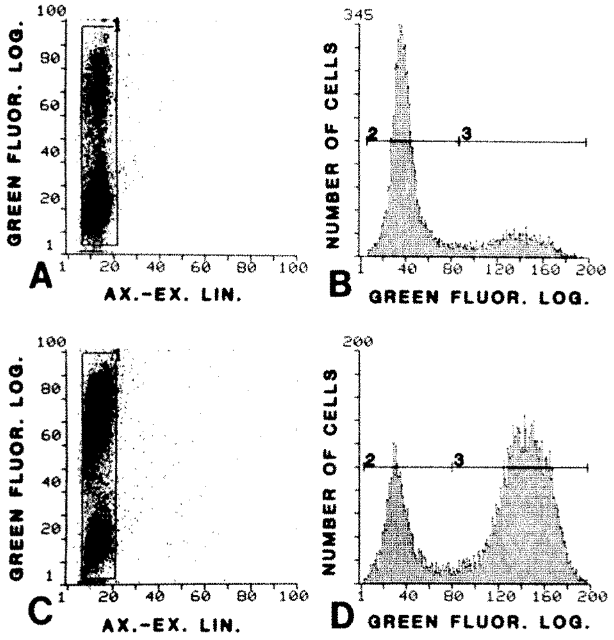

Fig 1. Flow cytometry analysis of red cells from an individual with heterocellular HPFH (panels A and B) and an individual with SS disease (panels C and D). Peripheral blood red cells were fixed, permeabilized, and then successively reacted with monoclonal anti-HbF and fluorescein-conjugated secondary antibody (see "Methods"). Panels A and C are cytograms, where the X axis equals linear-scale analysis of axial extinction of light (a measurement of cell shapes and sizes) and the Y axis equals logarithmic-scale analysis of right angle green fluorescence intensity. Reflected light from a helium-neon laser (630 nm) detects axial extinction, while an argon laser (480 nm) is used to detect right angle fluorescent emission (510 to 530 nm). Rectangular region 1 in panels A and C was defined to exclude cell doublets and encompasses erythrocytes of 4- to 10-μm diameter. Panels B and D are histograms depicting right angle green fluorescence (X axis) *v* cell number (Y axis). Panels B and D each represent 10,000 cells detected within region 1 from panels A and C, respectively. Region 2 in panels B and D demarcates a low-fluorescence non-F cell population, while region 3 demarcates a high-fluorescence F cell population. The proportion of cells in region 3 of panel B was 22.3% and in panel D was 72.1%.

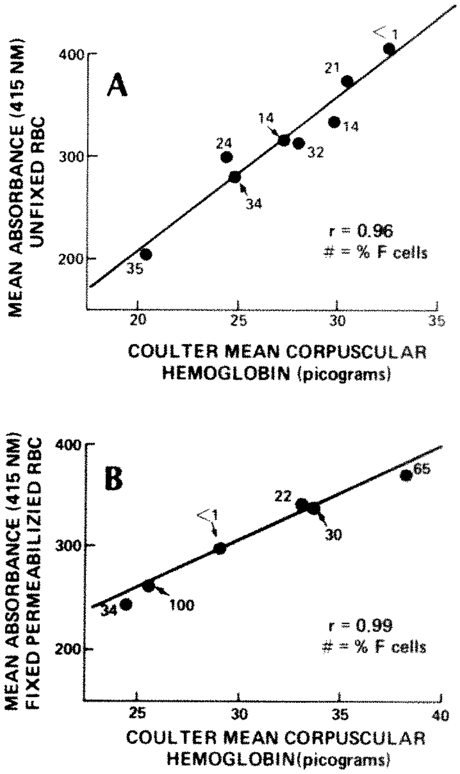

Fig 2. Mean absorbance of 415 nm light by 100 red cells compared to Coulter determined MCH for unfixed red cells (panel A) and for fixed, permeabilized red cells (panel B) reacted with anti-HbF antibody and fluorescent anti-antibody. Subjects included one pancellular HPFH (100% F cells), one heterozygote for heterocellular HPFH (21% F cells), one Fanconi's anemia (65% F cells), and two normal subjects (1% F cells); the remainder were SS subjects. Differences in slopes between panels A and B (1.5 and 0.9 respectively) are attributable to variation in light intensity and sensitivity of the photomultiplier. On any one day, however, values were reproducible.

body had been applied, leads to an equally good correlation (r = 0.99) between mean absorbance at 415 nm and the MCH as determined by Coulter assay. Thus, mean light absorbance from untreated and treated RBCs accurately reflect Coulter-derived values over a wide range of MCH and F cell levels.

MCH of F cells v non-F cells. Comparisons of the MCH measured by 415 nm absorbance of F cells *v* that of non-F cells were made in several groups of individuals. Figure 3 illustrates measurements of microscopically derived MCH in F cells *v* non-F cells for 11 individuals with SS disease. As seen, there was no difference over a wide range of MCH values between the MCH of F cells and non-F cells in any of the individuals studied ($P > 0.05$). As noted in Fig 3, three of these individuals were known to have associated alpha-thalassemia.

Five individuals with HbS-beta$^+$ thalassemia, and one with HbA-beta$^+$ thalassemia were analyzed using the same methodology (Fig 4). Figure 4 also illustrates comparisons of MCH of F cells *v* non-F cells in two normal subjects, two individuals with heterocellular HPFH with 12% and 21% F cells, and five untransfused individuals with congenital anemia, including one with Diamond-Blackfan anemia and four with Fanconi's anemia exhibiting F cell levels from 22% to 65%. In no individual was the MCH of F cells and non-F cells different ($P > 0.05$).

The degree of variability in hemoglobin content was similar for F cells and non-F cells. Among SS patients shown in Fig 3, the coefficient of variations (SD divided by mean) for hemoglobin content of F cells and non-F cells were no different (F cell: mean coefficient of variation = 20%, range 15% to 25%; non-F cells: mean coefficient of variation = 21%, range 16% to 30%). The same was true when comparisons of F cells and non-F cells were made for the heterogeneous group of individuals in Fig 4 (F cell: mean coefficient of variation = 17%, range 12% to 21%; non-F cells: mean coefficient of variation = 16%, range 12% to 25%). Furthermore, coefficients of variation were not correlated with the MCH or with F cell percentages in subjects described in either Fig 3 or Fig 4.

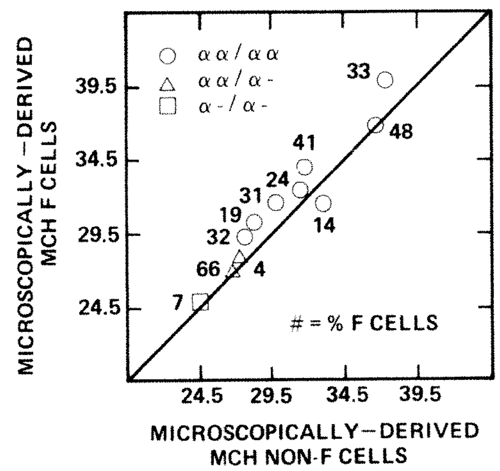

Fig 3. MCH for F cells and non-F cells in eight subjects with homozygote SS anemia and variable numbers of alpha-globin genes. F cells in these persons ranged from 7% to 48%. In this figure MCH was determined by assuming that the mean absorbance of 100 consecutive identified cells was proportional to the MCH determined by Coulter analysis. A standard line was then constructed, as in Fig 2B, and mean absorbance of 415 nm light for F cells and non-F cells was converted to MCH using this standard line. For each subject, 50 F cells were compared to 50 non-F cells regardless of the proportion of F cells present. The identification of F cells and non-F cells was made as described in "Methods."

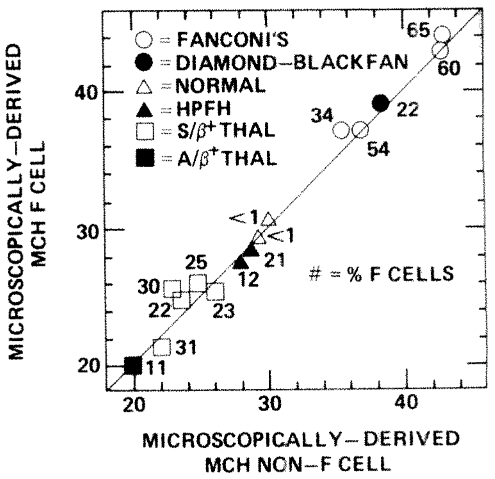

Fig 4. Comparison of the MCH of F cells and non-F cells in normals, in subjects with HbS-beta$^+$ thalassemia or HbA-beta$^+$ thalassemia, and in individuals with an elevated HbF production due to congenital anemia or heterocellular HPFH. MCH values and F cell identification were determined as outlined in "Methods" and Fig 3.

DISCUSSION

We and others have noted that in normal individuals and in individuals with hemoglobinopathies, HbF production precedes HbA production as cells mature from pronormoblast to reticulocytes.[13,14] Our previous work focused on the fact that HbF production is restricted to a subset of erythrocytes, F cells.[1,2,15] We wondered whether F cells made the same amount of HbA as non-F cells or whether early HbF production somehow restricted the amount of HbA produced in F cells. In this report we illustrate methodology that allows us to compare directly the hemoglobin content of F cells *v* non-F cells. We conclude from assays of a variety of subjects that F cells have the same MCH as non-F cells. Our data suggest that HbF and HbA production in adults are reciprocally controlled, regardless of the number of F cells produced. In addition, the fact that the MCH of F cells and non-F cells is identical suggests that the events that initiate HbF production within a red cell precursor indirectly lead to decreased HbA production in that same cell.

A reciprocal relationship between gamma- and beta-globin production can also be inferred from other studies. For example, decreased production of beta-globin and associated increases in gamma-globin are seen in some, although not all, patients with beta-thalassemia,[16,17] in individuals with pancellular (deletion) HPFH and delta-beta thalassemia,[18,19] and in two nondeletion forms of HPFH.[20,21] Heretofore, such reciprocity between gamma- and beta-globin gene production was gauged by measuring globin production in all hemoglobin-producing cells. Our data now establish that the same kind of reciprocal regulation exists within F cells from normal individuals and those with SS anemia, beta$^+$ thalassemia, or congenital anemia.

By what mechanism are HbF and HbA reciprocally controlled? Since HbF is produced earlier than HbA during erythroid development,[13,14] one might assume that the attainment of some absolute level of hemoglobin per cell would inhibit further hemoglobinization. This could result in decreased HbA production during late erythroid maturation within F cells. However, our data indicate that HbF and HbA remain reciprocally controlled over a wide range of MCH. Thus total hemoglobin content cannot, in itself, be the mediator of reciprocity between HbF and HbA production. A second possibility is that total hemoglobin production is controlled by the concentration of hemoglobin within cells, ie, MCHC. Indeed, early work by Yataganas[22] and Stohlman[23] indicate that erythroid progenitors stop dividing and hemoglobin biosynthesis falls away once a critical MCHC is attained. The importance of total hemoglobin concentration in erythropoietic development is underscored by the fact that the MCHC of eukaryotic red cells remains almost constant among evolutionarily divergent mammalian species.[24] Absolute hemoglobin content (MCH), on the other hand, is quite variable among species.[24] MCHC may therefore be the "hemostat" that curtails hemoglobin biosynthesis and may indirectly be responsible for reciprocal production of HbF and HbA. One might imagine that both F cells and non-F cells shut down hemoglobin production after some critical concentration of hemoglobin is attained. While the resulting F cells and non-F cells would have identical MCH, the early production of HbF would be later associated with a compensatory decrease in HbA.

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Contrasting Patterns of DNA Strand Breakage and ADP-Ribosylation–Dependent DNA Ligation During Granulocyte and Monocyte Differentiation

By Z. Khan and G.E. Francis

Previous studies have shown that structural changes in DNA, including the ligation of pre-existing DNA breaks and the opening and closure of new breaks, occur shortly after exposure of granulomonocytic precursors (CFU-GM) to granulocyte-macrophage colony stimulating activity (GM-CSA). Monocytic differentiation of CFU-GM is selectively inhibited by compounds known to inhibit the nuclear enzyme ADP-ribosyl transferase (ADPRT). Since this enzyme, which transfers ADP-ribose units to chromatin proteins, is known to activate DNA ligase, we attempted to determine whether ligation of one or both types of DNA break is required for monocytic differentiation. Breaks in DNA were examined using the nucleoid sedimentation technique in which DNA breaks cause loss of DNA supercoiling in nucleoids and concomitant changes in their sedimentation through neutral sucrose gradients. We here report that two distinct patterns of DNA strand breakage and ligation are associated with differentiation to the granulocyte and monocyte lineages. Monocytic inducers (phorbolester and vitamin D3) predominantly produce closure of pre-existing strand breaks, whereas granulocytic inducers (granulocyte colony stimulating activity, G-CSA; retinoic acid) cause opening and closure of new breaks. Only ligation of the pre-existing breaks is highly sensitive to inhibition by 3-methoxybenzamide (a potent ADPRT inhibitor), and only monocytic differentiation is impaired by addition of this compound. These findings suggest that DNA structural changes may be directly involved in granulocyte-macrophage switching.

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THE RIBOSYLATION of proteins by the nuclear enzyme ADP-ribosyl transferase (ADPRT) has been implicated as an obligatory event in differentiation in several eukaryotic cell systems.[1-4] This chromatin-bound enzyme transfers ADP-ribose units from NAD^+ to chromatin proteins forming homopolymers.[5,6] The enzyme is activated by DNA strand breakage,[7-9] and the ensuing ribosylation of chromatin proteins in turn activates DNA ligase.[10,11] It has been demonstrated that ADPRT is necessary for the repair of some types of DNA damage,[12,13] but it is not yet known whether the role of ADPRT in differentiation is linked to DNA ligation. ADP-ribosylation reactions are involved in a number of other cellular processes that might conceivably influence differentiation. Recent experiments in this laboratory[14,15] did, however, suggest that DNA ligation might be an essential step in monocytic differentiation. First, the exposure of bone marrow progenitor cells to granulocyte-macrophage colony stimulating activity (GM-CSA) is rapidly followed by an increase in ADPRT activity. Secondly, this is associated both with the closure of pre-existing DNA strand breaks and with the opening and closure of new breaks. Thirdly, the use of a variety of inhibitors of ADPRT preferentially inhibited monocytic differentiation of CFU-GM (as well as having the anticipated effect of delaying DNA ligation).

Since the early studies[14,15] had used a combined granulocyte-monocyte differentiation stimulus, the object of this study was to investigate whether inducers specific for granulocytic and monocytic differentiation behave differently with respect to the patterns of DNA strand breakage and/or ligation that follow induction to differentiation. We also wished to determine whether the monocytic differentiation induced by the pharmacologic agents phorbol myristate acetate (PMA) and vitamin D3 can, like the monocytic differentiation induced by GM-CSA, be inhibited by ADPRT inhibitors. The intention here was to establish whether agents acting by different mechanisms all require ADPRT.

MATERIALS AND METHODS

Bone marrow samples. These were obtained from healthy human volunteers with the approval of the Ethical Practices Committee of the Royal Free Hospital. Nonadherent low density bone marrow cells were prepared as previously described.[14]

Differentiation inducers. The following differentiation inducers were used: Phorbol myristate acetate (PMA) (Sigma Chemical, Poole, UK); 1,25-dihydroxyvitamin D3, all-trans retinoic acid (both gifts from Roche Products, Welwyn Garden City, UK); granulocyte-macrophage colony stimulating factors (GM-CSA) from either phytohemagglutinin-stimulated human peripheral blood leukocyte-conditioned medium (PHA-LCM) or unstimulated peripheral blood leukocytes (LCM); G-CSA from human umbilical cord conditioned medium (UCM; a gift of Dr T.C.M. Morris). PHA-LCM,[16] LCM,[14] and UCM[17] were prepared as described elsewhere and were used without further purification of the contained activities. It should be noted that although the term GM-CSA is used to refer to the activity derived from the former, we do not exclude the possibility that this material contains G-CSF, multi-CSF, and other biological activities.

Nucleoid sedimentation. The technique used was essentially that of Weniger[18] devised for the detection of low levels of DNA breaks induced by irradiation. The principle of this method is to lyse and strip cells of most of their protein in a high salt solution containing neutral detergents, which releases DNA in the form of nucleoids (highly ordered structures fixed by residues of membranes). Since the DNA in nucleoids exhibits a supercoiled structure, the presence of strand breaks increases the free rotation of DNA and therefore decreases the numbers of superhelical turns. This leads to a decrease in the sedimentation constant of the

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nucleoids. The band of nucleoids is made visible by staining with the fluorescent dye ethidium bromide. The advantage of this method is that it is more sensitive than most other techniques and also avoids the detection of breaks at apurinic sites, which are induced by alkaline conditions (required for alkaline sucrose gradients and the Birnboim and Jevcak technique used in our previous study).[15]

Nonadherent low density (<1.077 g · cm^{-3}) bone marrow cells (5 × 10^5 cells/mL) were exposed to the various differentiation inducing compounds for 20 to 120 minutes in the presence and absence of 5.0 mmol/L 3-methoxybenzamide (3-MB), washed once in Dulbecco's phosphate-buffered saline (PBS) at 4°C (without 3-MB), and resuspended in PBS at 2 × 10^7 cells/mL. Cells were lysed on top of a 15% to 30% linear (10 mL) sucrose gradient containing 2 mol/L NaCl, 10 mmol/L Tris-HCl pH8, 10 mmol/L EDTA Na_2, and 30 μg/mL ethidium bromide. The gradients were overlayered with 0.4 mL of lysis mixture: 0.1 mol/L Tris, 2 mol/L NaCl, 10 mmol/L EDTA pH 8, 0.5% triton X100, and 30 μg/mL ethidium bromide. Aliquots of 100 μL of the cell suspensions (2 × 10^6 cells) were layered onto the lysis mixture and left for 15 minutes at 20°C. Gradients were centrifuged for 60 minutes in a 6 × 14 SW rotor (MSE) at 75,000 g. The cellulose nitrate tubes were then illuminated at 366 nm, and the position of the fluorescent ethidium bromide/DNA band in each tube was marked. The ratio of the sedimentation distance of the DNA of control cells (which had not been exposed to differentiation inducer) to the sedimentation distance of DNA from treated cells was used to express results.

ADP ribosyl transferase assays. ADPRT was assayed in permeabilized, low density (<1.077 g · cm^{-3}), nonadherent bone marrow cells by incorporation of [^{3}H]-NAD (kindly provided by Dr W. Wish) into acid insoluble material, essentially as previously described.[14] Cells were exposed to differentiation inducers for 30 minutes prior and for 30 minutes during the permeabilization step of the assay (by incorporating an appropriate concentration of the inducing agent in the hypotonic solution). Cells were washed in Puck's saline A (5.4 mmol/L $MgCl_2$, 137 mmol/L NaCl, 4.2 mmol/L $NaHCO_3$), then resuspended into hypotonic solution for permeabilization (9 mmol/L HEPES pH 7.8, 5 mmol/L dithiothreitol, 5.5 mmol/L glucose [wt/vol], 2% dextran of approximate mol wt 110,000, 1.0 mmol/L EDTA, 4.5 mmol/L $MgCl_2$). After approximately 90% permeabilization (as detected by trypan blue), usually 30 to 40 minutes, a ninefold volume of isotonic buffer was added giving a final concentration of 1.4 × 10^7 cells/mL. The isotonic buffer contained 40 mmol/L HEPES pH 7.8, 130 mmol/L KCl, 2.5 mmol/L dithiothreitol, 2% (wt/vol) dextran, 225 mmol/L sucrose, 2 mmol/L EDTA, and 2.3 mmol/L $MgCl_2$. Triplicate samples of 300 μL of this cell suspension were incubated at 26°C with 50 μL of 140 μmol/L [^{3}H]-NAD$^+$ (26 Ci/mmol/L) in isotonic buffer. At 10 minutes the incubation was terminated by the addition of 0.5 mL of ice-cold 10% (wt/vol) trichloracetic acid containing 5 mmol/L NAD$^+$ and 2% tetrasodium pyrophosphate. For a positive control, permeabilized cells were treated with 20 μL of 2 mg/mL bovine pancreas deoxyribonuclease I (Sigma Chemicals, Poole, UK) in 10 mmol/L Tris HCl (pH 7.8) and 5 mmol/L $MgCl_2$. In addition, trichloracetic acid was added at time zero to triplicate samples of unstimulated cells to assess nonspecific binding. After 30 minutes at 0°C, the cell precipitate was filtered onto glass fiber discs using a Skatron cell harvester. The discs were washed five times with 2% (wt/vol) trichloracetic acid containing 5 mmol/L nicotinamide and 2% (wt/vol) tetrasodium pyrophosphate. The acid insoluble material was dissolved in hyamine hydroxide and radioactivity estimated by scintillation counting.

Bone marrow cultures. Nonadherent, low density bone marrow cells were cultured for seven days in 200 μL suspension cultures in microtitre plates and assessed for differentiation induction using dual esterase staining as previously described.[19] Cells were exposed to the differentiation inducers with and without 3-MB throughout the culture period. One millimolar 3-MB was used because higher levels can be toxic and also because this is a level known to significantly inhibit monocytic differentiation in GM-CSA stimulated cultures.[14] Appropriate diluent controls were used for unstimulated cells. With the differentiation inducers, since the cells also require a growth stimulus, we used 5% PHA-LCM. This was not, of course, used in the G-CSA cultures and was replaced by 5% LCM (as a source of GM-CSA) in some cultures. After the culture period cells were harvested by cytosedimentation and were stained for nonspecific and chloroacetate esterase activity.[14]

RESULTS

Figure 1 summarizes the results of experiments in which breaks in DNA were detected by loss of DNA supercoiling in nucleoids and their subsequent changes in sedimentation

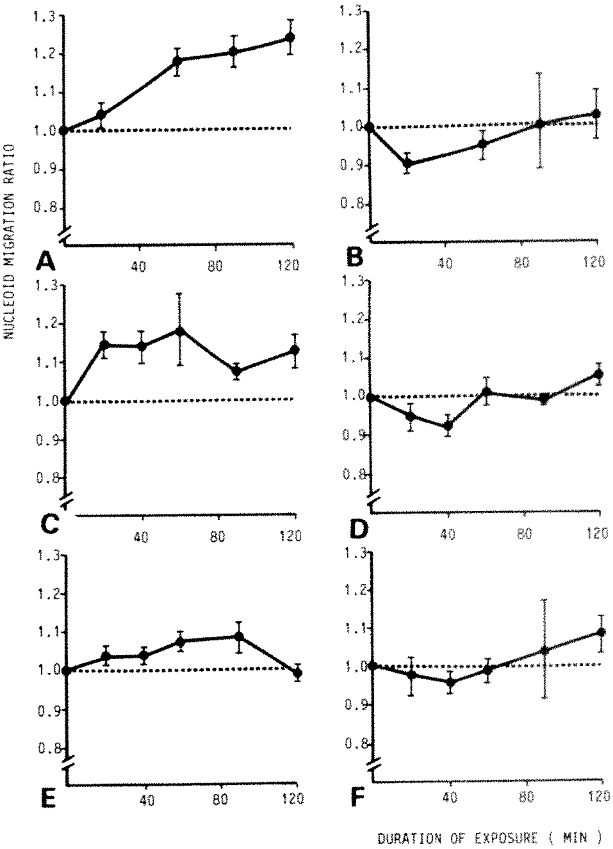

Fig 1. Migration ratios on nucleoid sedimentation gradients following treatment with physiologic and pharmacologic differentiation inducers. (A) GM-CSA (5% PHA-LCM or 5% LCM); (B) GM-CSA + 5.0 mmol/L 3-methoxybenzamide; (C) 10^{-9} mol/L PMA; (D) 10^{-6} mol/L retinoic acid; (E) 10^{-9} mol/L 1-25,dihydroxy vitamin D3; (F) G-CSA (50% umbilical cord-conditioned medium). Results are mean of four, four, five, six, five and two independent experiments for A through F respectively. With monocytic inducers (PMA and Vitamin D3) the increase in migration ratio indicates the closure of pre-existing strand breaks. With the granulocytic differentiation inducers (retinoic acid and G-CSA) there is opening of new strand breaks that are closed within 50 to 90 minutes of exposure. With a source of GM-CSA (PHA-LCM), closure of pre-existing strand breaks predominates in the absence of ADPRT inhibitor (3-MB), but in its presence there is opening and closure of new breaks similar to that seen with the granulocytic inducers.

through neutral sucrose gradients. Differences in sedimentation rate were expressed as a migration ratio relating the distance migrated by the test preparation to the distance migrated by nucleoids from the untreated control. Migration ratios >1 and <1 indicate DNA with less and more breaks respectively than present in the control preparation. As in previous experiments using a different technique, the exposure of cells to GM-CSA (obtained from two different sources, PHA-LCM and LCM) produced dual effects (Figs 1A and 1B): first, the closure of pre-existing strand breaks; secondly, in the presence of 5.0 mmol/L 3-methoxybenzamide, a potent inhibitor of ADPRT, there was also evidence of the opening and subsequent closure of new breaks. Unstimulated cells do not (as demonstrated in earlier studies) show such DNA breaks when exposed to 3-MB.

We next looked at the effects of two known inducers of monocytic differentiation (PMA and vitamin D3) and two inducers of granulocytic differentiation (retinoic acid and G-CSA). The results in Figs 1C through 1F show two distinct patterns of DNA strand breakage and ligation. The monocytic inducers predominantly show closure of pre-existing strand breaks, and the granulocytic inducers show opening of new strand breaks followed (within 50 to 90 minutes of exposure) by their ligation.

To assess whether both types of break require ADP-ribosyl transferase for their ligation, cells were exposed to both granulocytic and monocytic inducers of differentiation in the presence of 3-methoxybenzamide. Figure 2, panels A and C, shows that this agent effectively prevents the closure of pre-existing strand breaks. In addition, the nucleoid sedimentation profiles with PMA and vitamin D3 in the presence of 3-MB indicate that new strand breaks are also opening. These breaks are not seen without 3-MB because their effect on the nucleoid profile is masked by the closure of pre-existing strand breaks, this being the predominant effect. In contrast to inhibition of ligation of pre-existing breaks, some ligation of new breaks was evident (as an upward trend) in four of five profiles with 5 mmol/L 3-MB (Fig 1B and Fig 2A, B and D).

DNA breaks are known to activate the enzyme ADP-ribosyl transferase. Despite the different nucleoid sedimentation profiles, there was no gross difference (Fig 3) between the four different compounds with respect to the level of activation of ADPRT. Nor was there any difference between the two groups of compounds with respect to the kinetics of activation of ADPRT. Assays at an earlier time point (10 minutes) than that of Fig 3 also failed to show differences between the two groups (data not shown). In all cases, as one might expect, this was much lower than the activation produced by DNA damage (eg, nuclease-induced DNA strand breakages).

Contrasting effects of ADPRT inhibitor 3-methoxybenzamide on granulocytic and monocytic differentiation induced by the four compounds are shown in Fig 4. To avoid toxicity (since agents were incorporated throughout the culture period), lower concentrations of both the inducers and the ADPRT inhibitor were used than in other experiments. As in previous studies with GM-CSA, monocytic differentiation was inhibited, whereas granulocytic differentiation was spared or even enhanced. Thus the pharmacologic inducers behave in a similar way to GM-CSA.

DISCUSSION

The results of nucleoid sedimentation experiments confirmed our earlier finding[15] (using a fluorometric method[20] to

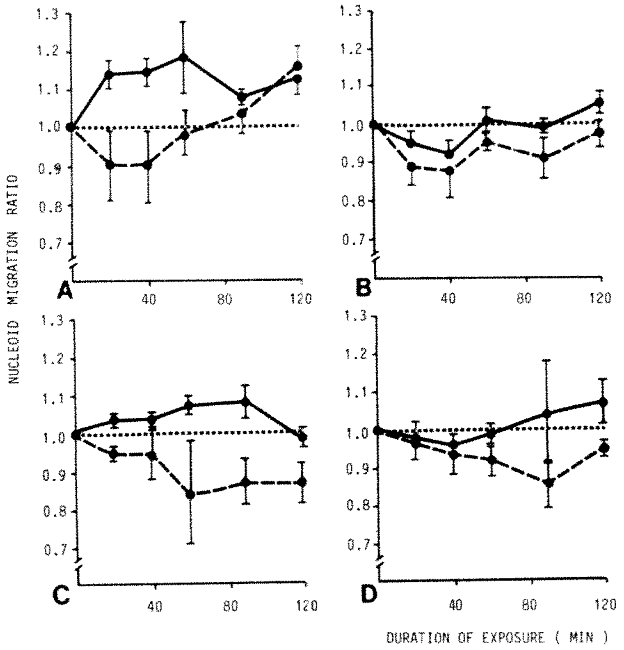

Fig 2. The effect of ADPRT inhibitor (5.0 mmol/L 3-methoxybenzamide) on nucleoid sedimentation after exposure to differentiation inducers. Dashed and solid lines show migration ratios with and without inhibitor respectively. Differentiation inducers are (A) 10^{-9} mol/L PMA; (B) 10^{-6} mol/L retinoic acid; (C) 10^{-9} mol/L 1-25,dihydroxy vitamin D3; (D) G-CSA (50% umbilical cord-conditioned medium).

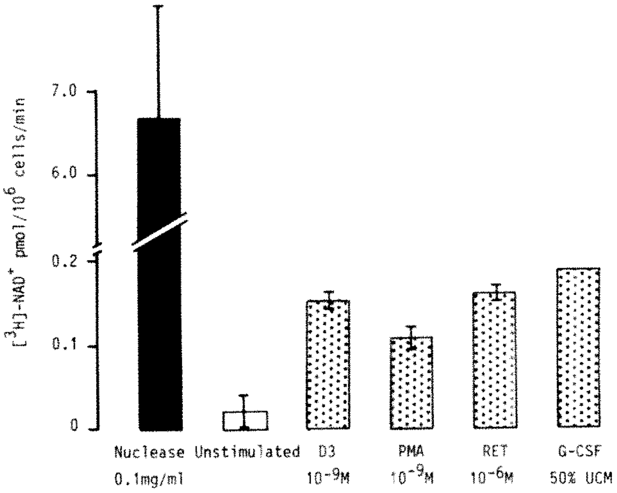

Fig 3. ADPRT activity levels (±SEM) after treatment with differentiation inducers for one hour (30 minutes before and 30 minutes during the cell permeabilization step) and with bovine pancreatic nuclease (positive control). 3-methoxybenzamide 2.5 mmol/L abolished all activity with each of the inducers (data not shown).

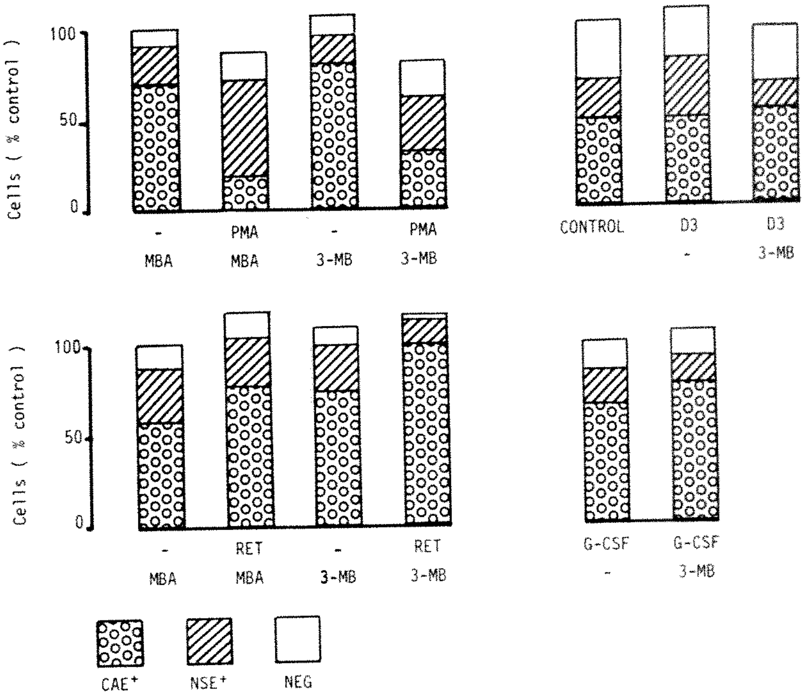

Fig 4. Effect of ADPRT inhibitor (1 mmol/L 3-methoxy-benzamide, 3-MB) on differentiation induction. The structural analogue 3-methoxybenzoic acid (1 mmol/L MBA), which is not significantly inhibitory for ADPRT, was used as a control. PMA 3 × 10^{-10} mol/L; Retinoic acid (RET) 10^{-7} mol/L; vitamin D3 10^{-9} mol/L; G-CSA 10% UCM. Cells were typed by dual esterase staining into:chloroacetate esterase positive cells (CAE$^+$ granulocytes); nonspecific esterase positive cells (NSE$^+$ monocytes); and negative cells (NEG). Results are representative examples of three experiments for each agent. Other doses of the same agents were also tested (data not shown). Inhibition of ADPRT by 3-MB reduced the monocytic differentiation in these cultures but not the granulocytic.

assess DNA strand breakage) that stimulation of bone marrow cells by GM-CSA is associated both with the closure of pre-existing strand breaks and with the opening of new breaks. To demonstrate the latter, the cells have to be exposed to an inhibitor of the nuclear enzyme ADP-ribosyl transferase, which inhibits the closure of pre-existing strand breaks (the predominant effect in GM-CSA stimulated cultures). The inhibitors of ADPRT not only affect the closure of DNA strand breaks induced by GM-CSA[15] but have an effect on the differentiation potential of the cells so treated.[14] Both in this and earlier studies, GM-CSA-stimulated bone marrow cells exposed to a range of ADPRT inhibitors show selective inhibition of monocytic differentiation while retaining the ability to differentiate to the granulocytic lineage.[14] Although the evidence for involvement of ADPRT in monocytic differentiation is indirect, no known property of the inhibitory compounds, other than their relative capacities to inhibit ADPRT, correlates closely with their capacity to inhibit monocyte formation.

Analysis of the response to agents that selectively promote either granulocytic or monocytic differentiation revealed two distinct patterns, with monocytic differentiation being associated predominantly with closure of pre-existing strand breaks and granulocytic differentiation being associated with the opening of new breaks. The shift in the pattern of nucleoid sedimentation seen when GM-CSA is used in the presence and absence of 5.0 mmol/L 3-methoxybenzamide not only mimics the two different nucleoid sedimentation patterns seen with monocyte and granulocyte inducers respectively but also produces a shift in the differentiation pattern observed. It therefore seems likely that the DNA structural changes reflected by the different patterns in the nucleoid sedimentation may well be directly involved in granulocyte-macrophage switching.

The changes in DNA supercoiling observed with G-CSA and vitamin D3 (Figs 1C and F) were less pronounced than for retinoic acid and PMA respectively (Figs 1C and D), and this could reflect differences in the intensity of the differentiation stimulus. The only divergence in pattern observed was the decline in migration ratio at 120 minutes with vitamin D3 not observed with PMA. Such a change could be due to cell damage by the agent.

The experiments shown in Fig 2 investigated the effect of ADPRT inhibitor (5 mmol/L 3-MB), added with the four differentiation inducers, on nucleoid sedimentation. With all four inducers there were shifted nucleoid profiles, indicating that the ligation of some, but not necessarily all, DNA breaks is dependent on ADPRT. In addition, the experiments with ADPRT inhibitor revealed that all five compounds (GM-CSA in Fig 1B; PMA, RET, D3, G-CSA in Fig 2A through D, dashed lines) induced the net opening of new strand breaks. This was not seen with the monocyte inducers and GM-CSA without 3-MB because it was masked by closure of pre-existing strand breaks causing a net increase in migration ratio. Since in four of five profiles with 3-MB there is an upward trend after the nadir in migration ratio, this suggests that at least some ligation occurs at 5 mmol/L 3-MB (twice the concentration required to inhibit all detectable enzyme activity after one hour of incubation). Whether this upward trend is due to the ligation of the new breaks just formed or to a shift in the balance between opening of new and ligation of pre-existing breaks cannot be established from these profiles. One can conclude, however, that ligation of new breaks (or of a subset of pre-existing breaks) is either ADPRT independent or much less sensitive to inhibition of 3-MB than the ligation of all or most pre-existing DNA breaks.

The findings in marrow cultures (Fig 4) suggest a simple interpretation of these results. 3-MB, even at 1 mmol/L, significantly inhibits monocytic differentiation induced either by PMA (3 × 10^{-10} – 3 × 10^{-9} mol/L) or by vitamin

D3 (10^{-9} mol/L); it also inhibits the residual monocytic differentiation in RET (10^{-7} – 10^{-6} mol/L) and G-CSA-stimulated cultures. In contrast, granulocytic differentiation was not inhibited but was even enhanced by 3-MB. Taken together, the nucleoid and culture findings suggest that monocytic differentiation is associated with and dependent on ADPRT-dependent ligation of pre-existing breaks. All the five inducers also cause the opening of new breaks, and since ADPRT inhibitors only significantly affect monocytic differentiation, one has to conclude that either (1) the ligation of new breaks (if it occurs) is not ADPRT mediated, or (2) in time it escapes the competitive inhibition of 3-MB, or (3) ligation is ADPRT mediated, but is not an essential step in differentiation to either lineage. On balance, in view of the nucleoid profiles obtained with 5 mmol/L 3-MB, we favor the first hypothesis.

The new breaks seem unlikely to be due to DNA damage. Sham-treated, unstimulated cells do not acquire breaks over this time scale.[15] Also, a consistent degree of damage is unlikely with five dissimilar agents. All the agents, including the pharmacologic inducers, directly or indirectly stimulate cell growth[21-23] prior to terminal differentiation. Although DNA synthesis does directly involve DNA strand breakage, this is unlikely to be the cause of the breaks seen here because of the rapidity with which they occur. The demonstration in recent studies that the DNA breaks induced by retinoic acid are protein associated (Francis, Berney, Khan, and North, unpublished) raises the possibility that DNA topoisomerases might cause the transient new DNA breaks and relaxation of supercoiling observed here. This might explain a DNA ligation step refractory to ADPRT inhibitors. Signal transduction in the epidermal growth factor system seems to involve DNA topoisomerase.[24-26] Involvement of DNA topoisomerases in the regulation of eukaryocyte gene expression is also suggested in other systems.[27]

Since ADPRT activity is increased by DNA strand breakage, enzyme assays were performed to determine whether the enzyme activity was higher with one or other types of inducer. The observation that the levels of enzyme activation are similar with the four compounds suggests that differences in enzyme activity are not responsible for the differences observed in behavior of nucleoids. In this and in our previous studies,[14,15] unstimulated cells (ie, cells that had neither been exposed to GM-CSA nor to a differentiation inducer) had very low levels of ADPRT activity. This suggests that the pre-existing breaks that close with monocytic differentiation are present in some form that does not activate the enzyme.

Why should the granulocytic inducers activate ADPRT if the breaks induced do not appear to require ADP-ribosylation for ligation? Ferro and Olivera[28] have suggested that ADP ribosylation may be important in regulation of DNA topoisomerase activity, specifically in the prevention of inappropriate ligation (eg, sister chromatid exchanges). If, as we suspect, DNA topoisomerases are responsible for the new DNA breaks, concomitant activation of ADPRT might also have a role in that reaction distinct from its apparent role in regulation of DNA ligation in monocytic differentiation.

This study contributes to the increasing body of evidence that DNA strand breakage and ADP-ribosyl transferase-mediated DNA ligation is involved in eukaryotic cell differentiation.[1-4,29-38] It is, as far as we are aware, the first report in which differentiation to two distinct but related cell lineages has shown different behavior with respect to DNA strand breakage and ligation. The present study indicates that in addition to a generalized involvement in differentiation, ADP-ribosylation may be specifically associated with lineage switch mechanisms, whereby pluripotent progenitor cells give rise to several lineages of differentiated progeny.

ACKNOWLEDGMENT

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Stromal Growth Factor Production in Irradiated Lectin Exposed Long-Term Murine Bone Marrow Cultures

By Thomas A. Alberico, James N. Ihle, Chi-Ming Liang, Helen E. McGrath, and Peter J. Quesenberry

Hematopoietic regulatory factors produced by adherent (stromal) cells in long-term murine bone marrow cultures have been investigated. Using an in situ double layer agar overlay system, we demonstrated that exposure of the stromal cells to 1,100-rad irradiation increased their activities in stimulating colony formation of FDC-P1, an interleukin 3 (IL 3)-responsive cell line. The colony-stimulating activities (CSAs) of the irradiated stroma also stimulated normal marrow cells to form granulocyte-macrophage, megakaryocyte, and mixed lineage colonies. Addition of the lectin pokeweed mitogen to the irradiated stroma increased the level of CSAs. The FDC-P1 CSA of the irradiated stroma was inhibited by antibodies directed against murine granulocyte-macrophage colony stimulating factor (GM-CSF) but not by those against murine IL 3. Stromal-derived CSA for marrow cells was also partially blocked by anti-GM-CSF antibodies, probably reflecting the presence of other CSAs such as CSF-1. This latter growth factor has been found to be present in conditioned media from Dexter stroma, but levels are not increased after irradiation or lectin exposure. Partially purified GM-CSF, like IL 3, stimulated FDC-P1 proliferation and granulocyte, macrophage, and megakaryocyte colony formation. These results indicate that the major terminal differentiating hormone elicited by irradiation or lectin exposure of murine marrow stromal cells is GM-CSF. This growth factor, along with CSF-1, can account for the differentiated progeny produced in this system: macrophages, granulocytes, and megakaryocytes.

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COLONY-stimulating activities (CSAs) are important for the proliferation of hematopoietic stem cells in in vitro clonogenic cultures.[1-3] Cytokines such as erythropoietin, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin 3 (IL 3), granulocyte CSF (G-CSF), and macrophage CSF (CSF-1) are known to have stimulating activities on their target cells.[4-10] Recently these cytokines have been purified, and recombinant DNA-derived proteins have been produced from bacteria and mammalian cells.[4-15] The functions and regulation of these cytokines in hematopoietic systems, however, have not been thoroughly evaluated.

Using a long-term murine bone marrow culture, Dexter and Lajtha[16] and Dexter et al[17] have developed a system that provides a useful in vitro model for exploring hematopoietic regulation. The Dexter system, which simulates the bone marrow microenvironment, is composed of a complex adherent layer formed by both hematopoietic cells and adherent nonhematopoietic (stromal) cells. Although this culture system supports prolonged production of granulocytes, megakaryocytes, and macrophages, early studies of the conditioned medium had shown little or only transiently detectable amounts of CSAs.[18] Using a double layer agar overlay system, Heard et al[19] improved the sensitivity of the assays and successfully demonstrated elaboration of CSA from the adherent cell population of long-term murine cultures. Subsequently, Gualtieri et al[20] have shown that after in vitro radiation of Dexter culture, CSAs are markedly increased. The elicited CSAs, however, have not yet been identified and characterized.

In this paper we reported that treatment with lectin pokeweed mitogen further increased the CSAs' elaboration by irradiated adherent stromal cells. The secreted CSAs were identified by using antibodies directed against known cytokines.

MATERIALS AND METHODS

Long-term bone marrow cultures. Long-term liquid murine bone marrow cultures were established as previously described.[21] Bone marrow obtained from the tibias and femurs of either ICR or BDF1 12- to 20-week-old female mice (Dominion Labs, Dublin, VA and Jackson Labs, Bar Harbor, ME, respectively) were used. Cultures were established in 25 cm^2 Corning tissue culture flasks either from single cell suspensions or by expelling the marrow from one tibia and one femur directly onto the flask. Alternatively, cultures were carried out in 35 mm^2 Corning tissue culture plates or 0.32 cm^2 96-well Costar flat bottom tissue culture microtiter wells from single cell suspensions. In all experiments, although variable volumes of Fishers's media, 20% horse serum, and 10^{-7} mol/L hydrocortisone were used, cell concentrations were established between 3 to 4 × 10^6 cells per cc. Cultures were incubated at 33°C with 5% CO_2. They were maintained with weekly demidepopulation and replenished with cell-free fresh media.

In vitro irradiation. After three weeks of growth (one experiment was carried out to four weeks), nonadherent cells were removed, and all cultures were totally depopulated. The stromal layer was then exposed to 1,100 rad, using a gamma-cell 40 cesium 137 irradiation unit (Atomic Energy of Canada Limited, Ottawa, Canada) at a rate of 123 to 126 rad per minute. Control unirradiated stromal layers were included in each experiment. In one series of experiments, stromal layers were exposed to varying levels of irradiation (250, 700, 900, 1,100, 1,500, and 2,000 rad). After exposing these cultures to irradiation, they were incubated another seven days at 33°C with 5% CO_2 and 95% humidity. After seven days of incubation postirradiation, all supernatant media was removed and the stroma was assessed directly for CSA. Alternatively (see below), condition media was harvested from these cultures and assessed for CSA levels.

Stromal assay for colony-stimulating activity. Stroma was assayed for CSA using the method described by Heard et al[19] and modified by Gualtieri et al.[20] Seven days postirradiation all supernatant media was removed from these cultures, and a double-layer

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agar system was employed to assay for CSA. Briefly, an underlayer of McCoy's 5A media with 2 mmol/L l-glutamine, 16 μg/mL L-asparagine, 8 μg/mL L-serine, 1 mmol/L sodium pyruvate, 1 × 10^{-4} mercaptoethanol, and 15% fetal calf serum, mixed in equal part with 1% agar, was added to these cultures (after all supernatant had been removed) and allowed to gel for 20 to 30 minutes. This layer separated the adherent stromal cells from the target cell population. In a number of these experiments, including variable-dose radiation experiments, potential stimulatory effect of pokeweed mitogen (GIBCO, Grand Island, NY) was examined by adding this lectin at a final concentration of 2.5 μg/mL to the underlayer of both irradiated and nonirradiated cultures. After allowing this underlayer to gel, an overlayer was then added containing McCoy's media with additives plus 0.3% agar, along with the target cell population. The target cell populations were either the IL 3-dependent cell line, FDC-P1,[22] or ICR or BDF1 murine marrow cells at a concentration of 0.5 to 1 × 10^5 cells/mL. Cultures were incubated for seven days at 37°C and 5% CO_2. After incubation all cultures were removed from the incubator, fixed with formalin, and, by using a dissecting microscope, colonies of 50 cells or greater were scored.

Bioassays of conditioned media. In experiments where the conditioned media was used to measure the CSA produced by the stromal cells, Dexter cultures were established and irradiated as described above. The adherent layers were then incubated in media with or without 2.5 μg/mL pokeweed mitogen for 24, 48, 96, 120, 168 hours. At these time points the conditioned media was collected and used as a stimulus in a single layer version of the agar culture system described above. Unconcentrated conditioned medium was added at various levels to stimulate both FDC-P1 and fresh murine marrow colony formation in the agar cultures. Media from Dexter flasks with no cells and with and without pokeweed mitogen at a concentration of 2.5 μg/mL served as controls.

Colony morphology. Colonies are classified as macrophage, granulocyte, macrophage/granulocyte, macrophage/monocyte (see legend for Table 1) and megakaryocyte. Soft-agar cultures were fixed in 10% formalin seven days after plating, and whole-agar slide preparations were then made from these cultures.[20] These slides were then stained for acetylcholinesterase and counterstained with hematoxylin. Megakaryocyte colonies were defined as aggregates of three or more acetylcholinesterase-positive cells, and mixed colonies were defined as colonies that had <90% of the predominant cell type. Degenerate colonies were scored when distinctive morphological features were absent.

Antibody blocking experiments with adherent feeder layer. Dexter cultures were established and maintained in Costar 96-well microtiter tissue culture plates, as described above, to carry out experiments that involved limited amounts of reagents (ie, antibody to IL 3). Initial concentrations in the Costar 96-well microtiter plates were 3 to 4 × 10^6 cells/mL with a volume of 0.3 mL per well. All wells were demidepopulated weekly with total depopulation and replacement with cell-free media at three weeks and exposure of the adherent layers to 1,100 rad. Underlayers and overlayers contained the same concentration of McCoy's media and agar as described above. A total of 0.22 mL was used in each well, with 0.12 mL in the underlayers and 0.1 mL in the overlayer. Additives were plated in the underlayer or added directly to the overlayer. Additives included pokeweed mitogen at a concentration of 2.5 μg/mL, antibody to IL 3 at 1 mg/mL, and/or control rabbit IgG at 1 mg/mL. Antibody to IL 3 and control rabbit immunoglobulin was prepared as described by Bowlin et al.[23] Antibody to IL 3 at a concentration of 1 mg/mL totally inhibited FDC-P1 incorporation of tritiated thymidine, whereas control rabbit IgG at the same concentration showed no inhibition.

Antibody blocking of CM. In experiments assessing anti-IL 3 antibody blocking of conditioned media (cm), target cell populations were cultured in 96-well microtiter or Terasake plates using the agar system previously described. These target cells included the IL 3-dependent cell line FDC-P1 or normal murine marrow cells. Similar experiments utilizing stromal-derived cm were carried out using antibodies to recombinant GM-CSF (see below).

Polyclonal antibodies against recombinant Escherichia coli-*derived murine GM-CSF.* Purified recombinant murine GM-CSF (200 μg/rabbit) in an emulsion formed by equal volume of normal saline and Freund's complete adjuvant was injected into New Zealand white rabbits (2 mL/rabbit) at multiple sites intradermally. The rabbit was boosted with the same amount of antigen in Freund's incomplete adjuvant twice at one-month intervals. Serum was obtained ten to 14 days after each boost. The anti-GM-CSF serum was partially purified by precipitation with 40% ammonium sulfate, followed by dialysis against phosphate-buffered saline (PBS), pH 7.2. The solution was then incubated at 56°C to inactivate complement. The preparation from 1 mL of rabbit serum generally yielded 10 to 15 mg of rabbit IgGs. These anti-GM-CSF antibodies neutralized the cell proliferative activity of recombinant murine GM-CSF while pre-immune serum showed no effect.

Assay of natural murine GM-CSF (GM-CSA-2) or recombinant GM-CSF for FDC-P1 colony-stimulating activity. GM-CSA-2 was partially purified from supernatants of concanavalin A (Con A)-activated splenic T lymphocytes.[23] Briefly, this involved removing BALB/c spleens, gently mashing them, and washing these cells in RPMI 1640. This suspension was cleansed of RBCs and was cultured with 5 μg/mL of Con A for 48 hours at 37°C. Cells were removed by centrifugation, and the supernatant was used for purification. The purification of the supernatant included ammonium sulfate precipitation with subsequent dialysis against a phosphate buffer. This dialysate was clarified by centrifugation at 10,000 RPM for 30 minutes and then applied to a diethyl aminoethyl (DEAE) cellulose column. The GM-CSA-2 activity was eluted from the column at 0.1 mol/L NaCl.[23] This GM-CSA-2 purified in this manner is serologically and biochemically distinct from IL 3 and is not inhibited by antibody to IL 3.[23] One unit of activity is defined as the amount of GM-CSA-2 that in 1 mL gives 50% maximal stimulation of a GM-CSA-responsive cell population. In a single-layer soft-agar system consisting of McCoy's 5A media plus additives and 0.3% agar (at a concentration of 80 U/mL), GM-CSA-2 was assayed for FDC-P1 CSA. These cultures were incubated for seven days at 37°C, 5% CO_2. After seven days they were removed from the incubator, fixed with formalin, and colonies of 50 cells or greater were scored. Alternatively, recombinant *E coli*-derived murine GM-CSF was tested.[24]

Sources of IL 3. IL 3 was purified to homogeneity from WEHI-3-conditioned media, as previously described.[10] Briefly, conditioned media from WEHI-3 murine myelomonocytic leukemia cells was concentrated on a 10,000-mol wt Amicon membrane. Ammonium sulfate fractionation was then used with a 50% to 80% fraction chromatographed on DEAE cellulose. IL 3, unlike GM-CSF, was not bound to the resin and was collected in the flow-through fraction. The flow-through fraction was concentrated and further purified by hydroxyl appatite column chromatography, followed by G70 Sephadex and high-pressure liquid chromatography.[10,25] The IL 3 preparations were then diluted in RPMI 1640 with 10% fetal calf serum and assayed for IL 3 using thymidine incorporation into the FDC-P1 indicator cell line, as previously described.[26] The preparation of IL 3 contained 0.2 ng/U of activity, with one unit of activity defined as the amount of IL 3 that in 1 mL gives 50% of maximal tritiated thymidine incorporation into FDC-P1 cells in culture.

Histochemical stains of stroma. Histochemical stains were used to evaluate the cytochemistry of the stromal cell population following XRT and pokeweed mitogen exposure. These included alpha naphthyl acetate (nonspecific) esterase, acid phosphatase, and alkaline phosphatase. Stromal cells were stained 48 hours after exposure to XRT and pokeweed mitogen. Over 500 cells were counted per 35-mm plate, and percent positivity was scored qualitatively 0 to 4+. Cells in the 2 to 4+ range were scored as positives.

RESULTS

In situ stromal assay for growth factor production. The capacity of nonirradiated and irradiated 3- to 4-week-old Dexter stroma to produce a growth factor capable of stimulating proliferation of FDC-P1 cells is shown in Fig 1. Total colonies of 50 cells or greater were scored at seven days. There was an 18.2-fold increase in the numbers of FDC-P1 colonies stimulated by the irradiated stroma as compared to the nonirradiated stroma. However, colony growth in this system did not approach that seen with IL 3-rich WEHI-3-conditioned media; in these preparations FDC-P1 colonies were too numerous to count. Pokeweed mitogen (PWM) stimulates IL 3 production from T lymphocytes. Thus we added this to the adherent Dexter cell underlayer to evaluate its effect on the irradiated and nonirradiated stromal growth factor production (Fig 2). Addition of pokeweed mitogen at a concentration of 2.5 μg/mL to nonirradiated stroma did not increase colony formation of the FDC-P1 cell line in the agar overlayers as compared to nonirradiated stroma without pokeweed mitogen. However, when the pokeweed mitogen was added to the underlayer of cultures with irradiated stroma, we found an 11.4-fold increase in colony numbers as compared to irradiated stroma without pokeweed mitogen. Different levels of irradiation were also assessed with and without the addition of PWM. As before, there is a marked increase in FDC-P1 CSA with the addition of PWM, with activity reaching a plateau at 700 rad (Fig 3). Although detectable, FDC-P1 CSA is lower without the addition of PWM, and there is a similar increase in FDC-P1 CSA with

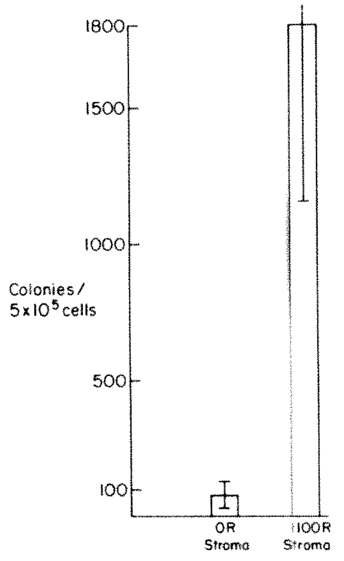

Fig 1. In situ stromal assay for FDC-P1 CSA from irradiated and nonirradiated adherent cells in Dexter culture. Adherent stromal layers from 3- to 4-week-old Dexter stroma were overlaid with 0.5% agar and McCoy's media, followed by a second overlayer consisting of 0.3% agar and media and the FDC-P1 target cell population (see Materials and Methods). CSA levels are expressed as the mean number of FDC-P1 colonies per 5 × 10^5 target cells ±1 SEM. These data are pooled from seven experiments. Two to four flasks or petri dishes were scored per group in each experiment. Abbreviations: OR = no irradiation; 1100R = 1,100 rad.

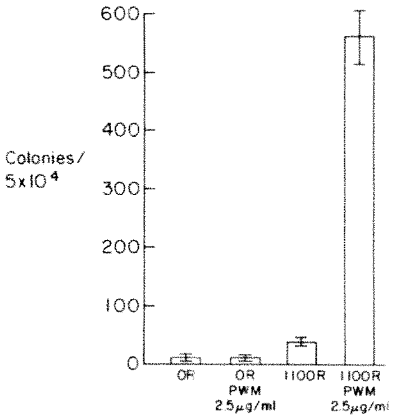

Fig 2. In situ stromal assay for FDC-P1 CSA from irradiated and nonirradiated adherent cells in Dexter culture after exposure to pokeweed mitogen (PWM). These experiments were carried out as described in the legend for Fig 1, except for the addition of 2.5 μg PWM to the 0 and 1,100-rad groups (see Materials and Methods). The FDC-P1 cells were plated at 5 × 10^4 in these experiments, as opposed to 5 × 10^5 in the experiments presented in Fig 1. CSA levels are expressed by mean numbers of FDC-P1 colonies/5 × 10^4 target cells ± 1 SEM. These data were pooled from three experiments, with three to four flasks or petri dishes scored per group in each experiment. Abbreviations: OR = no radiation; OR PWM = no radiation with pokeweed mitogen; 1100R = 1,100 rad; 1100R PWM = 1,100 rad with pokeweed mitogen.

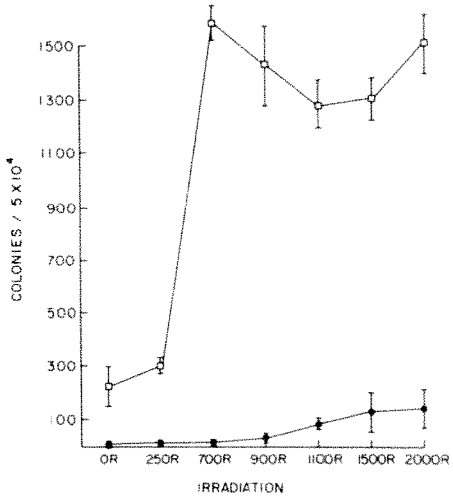

Fig 3. In situ assay for FDC-P1 CSA from Dexter culture adherent cells after exposure to varying irradiation levels with and without the addition of pokeweed mitogen (2.5 μg/mL). Abscissa represents escalating doses of radiation to which the adherent cells were exposed. This experiment was performed in a manner identical to that described in Fig 2, except for the varying levels of radiation. CSA levels are expressed as mean number of FDC-P1 colonies/5 × 10^4 target cells ± 1 SEM. This graph represents one of two experiments that were qualitatively similar. Three to four petri dishes were scored per group in each experiment. □, Irradiation + PWM; ●, irradiation alone.

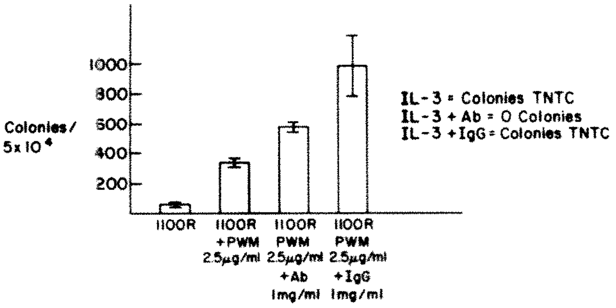

Fig 4. In situ stromal assay evaluating the effect of anti-IL 3 antibodies on FDC-P1 CSAs generated from irradiated pokeweed mitogen stroma. Antibody to IL 3 was prepared by rabbit innoculation as described by Bowlin et al.[23] Antibody (Ab) and control IgG were plated at a dose of 1 mg/mL along with pokeweed mitogen at a concentration of 2.5 µg/mL in the underlayer. Target cell population was the FDC-P1 cell line. Cultures were established in microtiter wells; these data represent two experiments, with four to six microtiter wells scored per group in each experiment. CSA levels are expressed by mean numbers of FDC-P1 colonies $\times 10^4$ target cells ±1 SEM. TNTC refer to cultures in which colonies were too numerous to count. Legend in left upper quadrant of figure indicates that IL 3 at a concentration of 4 ng/mL stimulated FDC-P1 colonies that were TNTC. Ab at 1 mg/mL totally blocked IL 3-stimulated FDC-P1 colony formation, whereas control rabbit IgG (1 mg/mL) did not inhibit colony formation.

irradiation alone, which plateaus at 1500 rad. These data suggested that the FDC-P1-stimulating activity from lectin-stimulated irradiated stroma might be IL 3. However, an antibody to IL 3 that effectively blocked pure IL 3 stimulation of clonal growth of FDC-P1 cells in a double-layer agar system did not block irradiated PWM stromal-induced colony formation; in fact, both the IgG control and anti-IL 3 antibody increased colony formation (Fig 4).

Assays of conditioned media. As stated previously, we (and others) have had difficulty in detecting significant levels of myeloid growth factors from Dexter culture conditioned media. We also have had repeatedly negative results when conditioned media was assayed for IL 3 using tritiated thymidine incorporation into the FDC-P1 line. It was for this reason that the in situ assay method was used. However, since high levels of activity were seen when the irradiated stroma was exposed to PWM, we again evaluated media conditioned by the irradiated lectin-induced stromal cells. Figure 5 demonstrates high levels of CSA in this conditioned media, stimulating clonal proliferation of both FDC-P1 and fresh murine BDF1 marrow cells. There was no stimulation of the FDC-P1 cell line by media conditioned by irradiated stroma without pokeweed mitogen, but cm from irradiated stroma exposed to pokeweed mitogen had increased levels of CSF for both marrow and FDC-P1 cells. However, the threshold for stimulation was lower for the murine-marrow target cell population compared to the FDC-P1 target cells. Figure 5 shows the effect of the conditioned media after 48 hours of culture. These results were confirmed in two other experiments, and the same phenomenon was noted at time points of 24 and 168 hours in one of these three experiments.

We have previously shown that CSF-1, as determined by radioimmunoassay, was present in cm from Dexter cultures and that the levels were not increased with irradiation.[20] We have repeated these studies here with similar results and have also shown that CSF-1 levels from irradiated or nonirradiated stroma were not increased by exposure to pokeweed mitogen (data not shown). In addition, we have cultured FDC-P1 cells with pure CSF-1 (courtesy of Dr Abdul Waheed and Dr Richard Shadduck) over a range of 78 to 10,000 U/mL and have shown that CSF-1 does not stimulate any clonal proliferation of these cells in soft agar culture.

Colony morphology. Table 1 shows types of murine marrow colonies observed in assays of conditioned media. The conditioned media obtained from irradiated lectin-treated stromal cells increased the numbers of all types of colonies, with up to a 70-fold increase in granulocyte-colony formation.

Assay of GM-CSA-2 for FDC-P1 colony-stimulating activity. Table 2 shows the effects of partially purified GM-CSA-2 from Con A spleen cm on FDC-P1 clonal growth in soft agar culture. GM-CSA-2 at a concentration of 80 U/mL stimulated FDC-P1 colony formation, but this did not approach the stimulation observed with IL 3. The blank containing McCoy's 5A media plus additives and agar

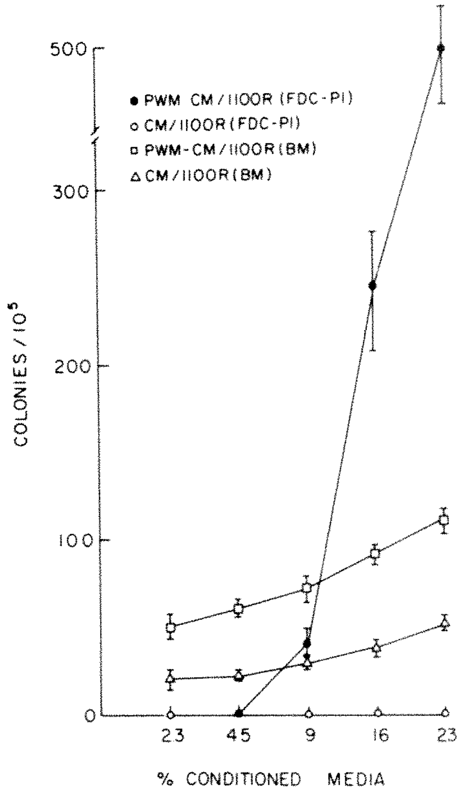

Fig 5. Assays of conditioned media from adherent cells of murine bone marrow cultures after exposure to irradiation and irradiation plus pokeweed mitogen. Concentration of pokeweed mitogen was 2.5 µg/mL. Media was collected after 48 hours of incubation and is a representative example of three separate experiments (see Materials and Methods). Colony-stimulating levels are expressed by mean numbers of both fresh murine marrow and FDC-P1 colony formation/10^5 target cells ±1 SEM. Three petri dishes were scored per group after seven days of incubation.

Table 1. Morphology of Colonies From Conditioned Media Assayed for Hematopoietic CSA Elaborated From Irradiated PWM-Exposed Dexter Stroma

	Percent Conditioned Media	Colony Morphology (colonies/10^5 cells)[†]					
		Granulocyte	Macrophage	Gran/Mac[‡]	Mac/Mono[§]	Meg[‖]	Deg[¶]
A. PWM CM	23	31.2 ± 2.3	22.6 ± 1.2	23.1 ± 6.0	15.2 ± 1.7	6.0 ± 3.4	2.6 ± 1.5
(1,100 rad)[*]	16	24.3 ± 1.6	26.1 ± 1.6	11.3 ± 2.3	11.6 ± 2.6	2.0 ± 1.1	2.6 ± 1.6
	9	25.3 ± 1.3	28.3 ± 3.0	13.7 ± 2.7	9.0 ± 2.6	.6 ± .3	3.0 ± .5
	4.5	19.5 ± 7.5	17.5 ± 1.5	14.2 ± 7.7	5.5 ± 1.5	.5 ± .5	1.5 ± 1.5
	2.3	14.3 ± 1.4	16.6 ± 3.0	4 ± 2.3	9.6 ± 2.7	0	9.1 ± 1.5
B. CM	23	8.3 ± 2.3	18.6 ± 7.5	7.6 ± 2.7	9.6 ± .8	0	9.6 ± 1.7
(1,100 rad)[*]	16	1.6 ± .9	14.6 ± 1.8	6.6 ± .8	9.0 ± 1.5	0	9.6 ± 1.3
	9	.33 ± .33	4.6 ± .8	2.1 ± .5	9.6 ± .9	0	20.0 ± 1.1
	4.5	.6 ± .6	1.6 ± .3	1.6 ± .6	4.3 ± 2.2	0	17.0 ± 1.3
	2.3	.5 ± .5	1.5 ± .4	.5 ± .5	7.5 ± 2.1	0	20.0 ± .8

[*] A and B represent media conditioned from adherent cells of Dexter culture at 48 hours after exposure to 1,100 rad ± pokeweed mitogen (PWM).

[†] Agar preps stained with acetylcholinesterase-hematoxylin. Numbers indicate absolute colony counts of one time point in one of three representative experiments. Three petri dishes were scored per group with counts ± 1 SEM.

[‡] Gran/Mac = Granulocyte/macrophage.

[§] Mac/Mono = Macrophage/monocyte = Typical macrophages intermixed with mononuclear cells with relatively high nuclear-cytoplasmic ratio and irregular cytoplasmic margins.

[‖] Meg = megakaryocyte.

[¶] Deg = Degenerative: Cells in these colonies were disrupted or fragmented or colony type not interpretable.

showed no colony formation. Recombinant GM-CSA also stimulated proliferation of FDC-P1 cells as assayed by ^{3}HtdR incorporation or clonal growth in agar (data not shown).

Effect of anti-GM-CSF antibodies on PWM/1,100 rad cm stimulation of FDC-P1 and murine marrow colony formation. Anti-GM-CSF antibody blocked the stimulation of both FDC-P1 and murine marrow colony growth by PWM/1,100 rad cm (Table 3). The inhibition was less for the marrow growth (53% ± 4.8%) but this is consistent with the presence of CSF-1, which is not inhibited by anti-GM-CSF (data not shown) and which does not stimulate FDC-P1 cells (see above). Thus the residual growth seen with murine marrow after GM-CSF blocking of PWM/1,100-rad cm is presumably due to CSF-1 in these preparations. The more complete inhibition of FDC-P1 proliferation by anti-GM-CSF suggests that GM-CSF is the primary stimulator of this cell type found in cm from PWM/1,100-rad stroma. In addition, an antibody to recombinant IL 3 was assessed for its ability to block FDC-P1 or murine marrow soft agar colony formation stimulated by PWM/1,100 rad cm in a fashion similar to that described for the anti-GM-CSF. No effect on marrow-(one experiment) or FDC-P1-(one experiment) stimulated growth was observed.

Table 2. Effect of GM-CSA-2 on FDC-P1 Clonal Growth in Soft-Agar Culture

Stimulus	FDC-P1 Colonies/10^5 Cells[*]
GM-CSA-2[†]	139 ± 16.7
IL 3[‡]	TNTC
Blank[§]	0

[*] In each group, three to four petri dishes were scored. These results are from three separate experiments.

[†] Preparations used in these studies were partially purified from supernatants of Con A-activated splenic lymphocytes (see "Methods"). One unit of activity was defined as amount of GM-CSA-2 in 1 mL, which was 50% of maximal stimulation of a GM-CSF-responsive cell population. GM-CSA-2 was plated at a concentration of 80 U/mL.

[‡] Interleukin 3 (IL 3) was used as a positive control in all experiments yielding colonies that were too numerous to count (TNTC). Preparations used in these studies had 0.2 ng of IL 3 per unit of activity. One unit of activity was defined as the amount of IL 3 that in 1 mL gives 50% of maximal tritiated thymidine incorporation into FDC-P1 cells in culture. IL 3 was plated at a concentration of 4 ng/mL.

[§] Blank media containing only McCoy's with additives (see "Methods") and 0.3% agar were used as negative controls and yielded no clonal growth.

Histochemical stains of stroma. Histochemical stains of irradiated stroma with and without exposure to PWM at 2.5 μg/mL showed no shift in cell types. We have previously reported on the staining characteristics of stroma exposed to 950 rad,[20] and the results with 1,100 rad alone were similar to our previously reported data except for a higher percentage of alkaline phosphatase positive cells in the present study.

DISCUSSION

Our present studies show that radioresistant adherent cells from murine Dexter cultures produce growth factors that stimulate FDC-P1 cells and murine marrow granulocyte-macrophage-megakaryocyte progenitors to proliferate.

The growth factor acting on FDC-P1 cells, which is increased by stromal irradiation and lectin exposure, appears to be GM-CSF. While CSF-1 is present in conditioned media from normal stroma, its levels are not increased by irradiation and/or lectin exposure, and, as noted above, pure CSF-1 does not stimulate FDC-P1 proliferation. The anti-GM-CSF antibody used in these studies blocks PWM/1,100-rad cm stimulation of FDC-P1 cells but does not block CSF-1 stimulation of marrow growth. Finally, we have confirmed the results of Hapel et al[27] showing that GM-CSF (recombinant or partially purified from Con A spleen cm) stimulates FDC-P1 proliferation as assessed by clonal agar culture or tritiated thymidine incorporation in liquid culture. The fail-

Table 3. Effect of Anti-GM-CSA on PWM/1,100 Rad Cm Stimulation of FDC-P1 and Bone Marrow Colony Formation

FDC-P1 cells‡

Stimulus	Antibody/Pre-immune Serum*	Colony (>10 cells)/ 2,000 cells
PWM/1,100 rad (31% final concentration)	Pre-immune serum	46.7 ± 5.4
	anti-GM-CSF	2.7 ± .7
GM-CSF (0.3 ng/mL)	Pre-immune serum	74.4 ± 4.7
	anti-GM-CSF	0.1 ± 0.1

ICR marrow cells§

Stimulus	Antibody/Pre-immune Serum†	Colony (>50 cells)/ 10,000 Cells
PWM/1,100 rad (33.3% final concentration)	Pre-immune serum	38.5 ± 3.5
	anti-GM-CSF	23 ± 5
GM-CSF (0.3 ng/mL)	Pre-immune serum	22 ± 1
	anti-GM-CSF	1.5 ± 1.5

*Final dilution of antibody/pre-immune serum was 1:6.

†Final dilution of antibody/pre-immune serum was 1:100. FDC-P1 10 cell colonies in Terasaki plates were counted at day 4 and BDF1 murine marrow colonies (>50 cells) at day 8 of culture.

‡The FDC-P1 data is from one experiment with nine replicate wells. This was confirmed in two separate experiments testing two different PWM/1,100-rad cm. The mean % inhibition (±1 SEM) of PWM/1,100-rad cm stimulated FDC-P1 colony formation caused by anti-GM-CSF was 87.3 ± 7. In the third experiment, growth was scored on a scale of 4+ (colonies so numerous that accurate counting was very difficult) to 0 (no growth). PWM/1,100-rad cm stimulated 4+ growth and exposure to anti-GM-CSF reduced this markedly to 0.5 to 1+ (few colonies).

§The data on ICR marrow is from one experiment utilizing 24-well Linbro plates and assessing replicate wells. This was confirmed in three other separate experiments using both BDF1 and ICR marrow cells and assessing two different lots of PWM/1,100-rad cm. The mean % inhibition (± are SEM) of PWM/1,100-rad cm stimulated colony formation caused by anti-GM-CSF for all experiments was 53 ± 4.8 (four experiments, six separate assessments).

ure of antibodies to IL 3 from two separate sources to block PWM/1,100-rad cm stimulation of FDC-P1 cells indicates that the FDC-P1 growth factor is not IL 3. Altogether these observations establish that GM-CSF is the FDC-P1 stromal-derived growth factor that increases after irradiation or lectin exposure.

These studies with FDC-P1 cells also indicate that the effects seen are probably direct and not mediated via accessory cells, since FDC-P1 cells do not proliferate in the absence of growth factors, and cm from FDC-P1 cells does not stimulate their own growth. In addition, we have shown here that PWM does not directly stimulate FDC-P1 growth (negative controls for Fig 2, data not presented).

The stromal growth factor for murine marrow, which is increased in the 1,100-rad and PWM/1,100-rad groups is probably also GM-CSF. CSF-1 is present in cm from Dexter culture but not increased by irradiation or lectin exposure. Anti-GM-CSF partially blocks PWM/1,100-rad cm marrow stimulation (53% ± 4.8% inhibition, Table 3), the residual marrow growth presumably being due to the presence of CSF-1. Consistent with this hypothesis are the preliminary observations that anti-CSF-1 antisera block PWM/1,100-rad cm stimulation of murine marrow colony formation by approximately 30%, while we have previously reported that marrow colony stimulation by cm from 950-rad stroma was inhibited from 46.6% to 92.4%[20] by exposure to anti-CSF-1 antisera. These data suggest that the primary CSA for murine marrow increased by lectin exposure is GM-CSF. In addition, colony formation stimulated by stromal-derived growth factors includes granulocyte, macrophage, megakaryocyte, and mixed lineage colonies, a spectrum of colony formation seen with either IL 3 or partially purified GM-CSF.[28] The anti-IL 3 studies cited above with FDC-P1 cells, a very sensitive IL 3 assay cell, plus the single experiment with marrow cells in which anti-IL 3 antisera did not block PWM/1,100-rad cm stimulation of marrow cell proliferation indicates that IL 3 is not the stromal-derived marrow stimulatory growth factor. Thus a radioresistant stromal cell appears to be stimulated by lectin exposure to release GM-CSF, which stimulates both FDC-P1 and a range of marrow progenitors. Finally, it should be noted that GM-CSF (with or without CSF-1) is capable of stimulating differentiation of all the differentiated hematopoietic lineages seen in Dexter marrow cultures: macrophages, granulocytes, and megakaryocytes.[28]

The identity of the radioresistant cell type generating these in vitro growth factors remains speculative. Two critical adherent cell types capable of supporting liquid culture hematopoiesis are seen after in vitro[20,29] or in vivo irradiation.[30] One cell type has the phenotype of a macrophage (phagocytic, acid phosphatase, and nonspecific esterase positive), while the other cell is a large alkaline phosphatase-positive epitheliod cell. Both cell types were Factor VIII negative and a small (<3%) fraction of cells did not fit either phenotype. In these studies we found no apparent shift in cell types with lectin exposure (Table 4). Thus the lectin responsive GM-CSF-producing cell types seem most likely to be either macrophages or the large alkaline phosphatase positive cells.

Previous studies from our laboratory have suggested that increases in myeloid stimulatory factors seen with irradiation of Dexter culture adherent cells may be due to removal of the negative feedback exerted by mature myeloid cells or removal of a population of cells that rapidly bind, utilize, or degrade these growth factors.[20] As with previous studies, our data on radiation dose response suggest an inverse correla-

Table 4. Results of Stromal Histochemical Stains Day 2 Post-XRT (% positive cells)†

Stain*	1,100 rad	1,100 rad + PWM (2.5 μg/mL)
Alpha naphthyl acetate (nonspecific esterase)	55	50
Acid phosphatase	95	97
Alkaline phosphatase	34	34

*Results were derived from one experiment, evaluating the stroma at day 2 post-XRT and PWM exposure. Eight hundred cells were scored for each stain.

†Cells stained at 2 to 4+ intensity on a scale of 0 to 4+ were scored as positive.

tion with FDC-P1 growth factor production and total amount of mature granulocytic elements present in the stromal layer.

The mechanism by which pokeweed mitogen stimulates stromal cells to produce increased levels of GM-CSF is not known. It seems probable that pokeweed mitogen selectively binds and stimulates a specific cell type remaining in the stromal layer after the exposure to irradiation, thereby increasing levels of GM-CSF. Alternatively, the pokeweed mitogen may act by eliminating an inhibitory phenomenon that may also increase colony formation. Studies by Gualtieri et al[20] demonstrated that conditioned medium from normal or irradiated stroma (without pokeweed mitogen) did not show differing levels of inhibitors. Also, in preliminary studies using conditioned media from irradiated cultures with and without pokeweed mitogen, we have found that when adding either of these conditioned media to purified CSF-1 there is no inhibition of growth. In fact, the conditioned medium from pokeweed mitogen-stimulated irradiated stroma, when added to pure CSF-1, actually increases both size and numbers of hematopoietic colonies, suggesting that a synergistic activity may be present in this conditioned medium. We have recently described an adherent cell line derived from murine Dexter cultures, termed TC-1,[31] which produces a CSF-1-dependent synergistic activity. At present it is uncertain whether this latter synergistic bioactivity may in part be responsible for the increased FDC-P1 and marrow colony formation seen with cm from irradiated lectin-exposed stroma.

In summary, we have shown that a radioresistant cell present in the adherent layers of long-term Dexter cultures responds to the lectin pokeweed mitogen by generating GM-CSF. CSF-1 is also produced by these adherent cells. These two regulators are sufficient to account for the differentiated hematopoietic progeny seen in this culture system: macrophages, granulocytes, and megakaryocytes.

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Vitamin B12-Responsive Neonatal Megaloblastic Anemia and Homocystinuria With Associated Reduced Methionine Synthase Activity

By Lynnette J. Hallam, Margaret Sawyer, Arthur C.L. Clark, and Martin B. Van Der Weyden

We present findings on an infant with neonatal megaloblastic anemia, homocystinuria, and neurologic dysfunction that included developmental delay and tonic seizures. There was no methylmalonic aciduria. Cyanocobalamin therapy was accompanied by complete hematologic and neurologic recovery, diminished homocystine excretion, and subsequently normal neurologic development. Cultured fibroblasts and lymphoblasts showed a reduced methionine synthase activity and a growth requirement for methionine. Cobalamin incorporation by the patient's lymphoblasts was normal, but the proportion of cellular methylcobalamin in the patient's lymphoblasts and fibroblasts were markedly reduced and that of adenosylcobalamin normal. The reduced methionine synthase activity was independent of assay reducing (thiol) conditions, but normal levels of activity accompanied culture of the patient's lymphoblasts in medium with markedly increased cobalamin concentration. The characteristics of the reduced methionine synthase of our patient differ significantly from that of the previously described infant with cobalamin E disease and suggest that genetic heterogeneity may characterize this mutation.

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IN HUMAN CELLS cobalamin (Cbl) occurs predominantly as either adenosylCbl or methylCbl. These cobalamins mediate reactions catalyzed by methylmalonylCoA mutase (adenosylCbl) in the generation of succinylCoA from methylmalonylCoA and that of methionine synthase (methylCbl) in the synthesis of methionine from homocysteine.[1]

Neonatal megaloblastic anemia consequent to inherited defects of cobalamin metabolism occurs either with a deficiency of the carrier protein transcobalamin II,[2] an obligatory mediator for cellular uptake of cobalamin, or with mutations leading to impaired intracellular synthesis of both adenosylCbl and methylCbl.[1] The latter states are accompanied by methylmalonic aciduria and homocystinuria as these two end products reflect intermediates normally metabolized by the cobalamin-dependent reactions in man. Recently Schuh and coworkers[3] described an infant with developmental delay, megaloblastic anemia, and homocystinuria but not methylmalonic aciduria who responded favorably to hydroxoCbl. The molecular basis for this syndrome was suggested to be failure of reduction of cobalamin bound to methionine synthase. In vitro findings included decreased fibroblast methylCbl but not adenosylCbl content and unique properties for methionine synthase, which exhibited normal activity at optimal but not at low assay reducing (thiol) conditions.[4] We describe here another infant with neonatal megaloblastic anemia and homocystinuria with reduced fibroblast and lymphoblast methionine synthase activities. This reduced activity, in contrast to that of the patient previously described, is independent of assay thiol concentration but dependent in part on the cobalamin levels of culture medium. These findings suggest that considerable genetic heterogeneity may characterize this newly described mutation of cobalamin metabolism.

CASE REPORT

The patient, a Caucasian male infant, was referred at the age of 10 weeks for investigation of anemia, hypotonia, and developmental regression. He was the first born of unrelated parents who had had no fetal losses. He was born at 38 weeks gestation, birth weight 3,180 g, and was in good condition at birth. His parents stated that he was never a very responsive baby but fed successfully from the breast and smiled and responded to his parents at about six weeks. At that time he had a febrile illness with diarrhea and vomiting lasting about one week, after which he became unresponsive and unsmiling. On admission to the hospital at 10 weeks he was a pale, floppy, unresponsive infant, height 59 cm, weight 4,730 g, and head circumference 39.3 cm. The liver was palpable 3 cm below the costal margin, the spleen not palpable, and lymph nodes not enlarged. Despite his generalized hypotonia, deep tendon reflexes were present and symmetrical, and no other neurologic abnormalities were noted. However, during the subsequent three weeks he developed truncal spasticity with head retraction and spine extension when disturbed, and on the fifth hospital day he had a generalized seizure. Three further seizures followed until he was given phenobarbitone, after which these ceased. Investigations on admission revealed anemia with hemoglobin 7.7 g/dL, hematocrit 21%, MCV 101 fl, MCH 35.5 pg, MCHC 53.2 g/dL, reticulocytes 3.5%, obvious anisocytosis, macrocytosis and poikilocytosis. The WBC was $8.6 \times 10^9/L$, neutrophils 27% with hypersegmentation, lymphocytes 71%, eosinophils 2%. Platelets were $719 \times 10^9/L$. Bone marrow was markedly cellular and megaloblastic. High voltage electrophoresis of the urine showed greatly increased homocystine and increased serine, alanine, leucine, and ethanolamine. Methylmalonic acid was not detected, and gas liquid chromatography showed a normal organic acid pattern. Plasma amino acid analysis was not performed before treatment. Two weeks after the first dose of vitamin B12, no homocysteine was detected in the plasma while methionine was 4 µmol/L (n, 5 to 34). Red cell folate was 1,280 nmol/L of packed cells (n, 230 to 2,200), serum folate 41 nmol/L, and B12 117 pmol/L (n, 160 to 500). Liver and renal function tests were normal. Cerebral ultrasound examination showed no abnormality, and an EEG showed medium voltage

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with no consistent asymmetry, focal, or epileptiform features. His mother's serum folate and B12 were normal, and she had no antibodies to intrinsic factor. Transcobalamin II deficiency was excluded by normal findings for patient's serum mediated 57Co-cyanocobalamin uptake by normal lymphoblasts and serum 57Co-cyanocobalamin elution pattern with gel chromatography. One week after admission treatment was begun with both folate and B12, the latter as 1,000 μg of cyanocobalamin intramuscularly (IM) daily. Reticulocytes peaked at 23% on the fifth day, and there was progressive rise in hemoglobin and return toward normal of red cell changes. On treatment his general health improved, his muscle tone returned to normal, and there were no further seizures. Homocystine was no longer detected in his urine. Anticonvulsants were discontinued at six months, and B12 was reduced to 1,000 μg twice a week at 12 months. At 2 years folic acid was discontinued. Developmental assessment at 3 years is within normal limits in all modalities. A female sibling was born 2 years later. She was physically and hematologically normal at birth. At 6 weeks she had a Hb 12.7 g/dL, MCV 100 fl, with normal red cell morphology. She made good progress until her sudden cot death at 9 weeks. Post mortem examination failed to reveal the cause of death. Attempts to establish Epstein-Barr virus (EBV)-transformed lymphoblasts from this individual were not successful.

MATERIALS AND METHODS

Cell lines. Human skin fibroblasts from the patient and normal individuals were routinely grown in Eagle's minimum essential medium (CS Laboratories, Parkville, Australia) with 10% fetal bovine serum (FBS Flow Laboratories, Australia). EBV-transformed lymphoblast cell lines were established from the patient, mother, father, and normal individuals as described previously.[5] All lymphoblast cell lines were maintained in exponential growth in RPMI 1640 medium (Flow Laboratories) supplemented with 10% FBS. The concentration of methionine under normal culture conditions is 0.1 mmol/L (15 mg/L) and vitamin B12 3.7 nmol/L (5 μg/L). In experiments with modifications of concentration of vitamin B12, folate, or methionine in the culture medium, cells were grown in a modified RPMI 1640 medium (K-C Laboratories, Kansas City) with the appropriate vitamin or amino acid added at the indicated concentration. Fibroblast and lymphoblast growth studies were initiated at a cell density of 2×10^5/mL and 5×10^5/mL respectively with the indicated culture medium conditions, and cell counts were performed as previously described at 96 hours using trypan blue exclusion as the index of viability.[6]

Fibroblasts were harvested by treatment with 0.25% trypsin (Flow Laboratories) for one minute at 37°C and subsequent inactivation with FBS.

Enzyme assays. Lymphoblasts or confluent fibroblasts were harvested as described, centrifuged at 1,000 g for 10 minutes at 4°C, and washed thrice with phosphate-buffered saline (PBS). Extracts of cells in appropriate buffers, usually 10 mmol/L Tris HCL pH 7.4 were performed by either freeze thawing in liquid nitrogen or by sonication. Cell extracts were centrifuged at 1,500 g for 10 minutes at 4°C and supernatant used for assay. Protein was determined using bovine serum albumin as standard by the method of Lowry et al.[7]

Thymidylate synthase,[8] serine hydroxymethyltransferase[9] and 5,10-methylene tetrahydrofolate (THF) reductase[10] were measured as previously described. Methionine synthase was measured by the method of Kamely et al with minor modifications.[11] Activity was determined by the formation of 14C-methionine from 14C-5 methyl THF (5-MTHF) in the dark. The standard reaction mixture contained in a total volume of 0.2 mL: 130 mmol/L sodium phosphate buffer pH 7.4 (Ajax Chemicals, Australia), 200 mmol/L B-mercaptoethanol (BDH Chemicals, Australia), 275 μmol/L S-adenosyl methionine (SAM) (Boehringer Mannheim, Germany), 50 μmol/L methylcobalamin (MeCbl) (Sigma Chemicals, St Louis), 500 μmol/L DL-homocysteine (Sigma Chemicals, St Louis), prepared just before use from the thiolactone derivative, 600 μmol/L (14C) 5-MTHF 2 μCi/μmol (Amersham, England) and 150 to 160 μg of cell extract. The assay control was minus the addition of MeCbl and SAM, and activity obtained with the omission of MeCbl was that of the holoenzyme. Incubation was in the dark at 37°C for 60 minutes. The reaction was terminated by adding 0.8 mL of ice-cold water. The mixture was passed through Bio-Rad AG-1X8 columns (Bio-Rad Laboratories, Richmond, CA), and the columns were washed with an additional 1.0 mL of water. The radioactivity in the pooled effluent was determined in a liquid scintillation counter. Enzyme-specific activities are expressed as nmol of product formed/hour/mg protein. Methionine synthase was assayed routinely under aerobic conditions as described above but was also determined anaerobically by flushing the reaction mixtures with nitrogen, which were then sealed during the incubation.

Intact cell assays. Incorporation of 14C-5MTHF into acid precipitable material was performed according to the method of Mellman et al,[12] and incorporation of 14C-propionate (Amersham, England) into acid precipitable material was as described by Willard et al.[13] Incorporation of 57CO-cyanocobalamin (Amersham) 0.1 ng/mL was performed at two hours and 72 hours as described by Mellman et al[14] and for fractionation of intracellular cobalamin cells were extracted according to the method of Linnell et al[15] and cobalamins separated on a SP Sephadex C-25 column (Pharmacia, Sweden) in the dark, as previously described.[4]

RESULTS

Methionine synthase activity in patient's cells. Altered cellular cobalamin metabolism in the patient's cells was explored by determining the incorporation of radioactivity from 14C-labeled propionate and methyl THF into protein of patient and control lymphoblasts precipitated by trichloroacetic acid. Incorporation by patient's cells of radioactivity from 14C-propionate was normal, but that from 14C methylTHF was significantly reduced (Table 1), suggesting a defect of methionine synthesis without an abnormality in the function of methylmalonylCoA mutase. The level of activities of enzymes of patient's lymphoblasts that are involved in thymidylate synthesis, namely thymidylate synthase, 6.5 ± 1.4 nmol/hour/mg protein (mean, SE) serine hydroxymethyltransferase 257 ± 61 nmol/hour/mg protein, or that of 5,10 methylene THF reductase 2.3 ± 0.3 nmol/hour/mg protein were comparable to the corresponding values of those activities in control lymphoblasts: 6.7 ± 0.6, 386 ± 36, and 1.8 ± 0.2 nmol/hour/mg protein. Methionine synthase was measured in patient and control cell extracts with and

Table 1. Incorporation of 14C-Propionate and 14C-MethylTHF Into TCA Precipitable Material of Control and Patient's Lymphoblasts

Cells	14C-propionate* 100 μmol/L; 14 μCi/μmol	14C-methylTHF† 25 μmol/L; 25 μCi/μmol
Control	825 ± 163	479 ± 31
Patient	788 ± 190‡	154 ± 26§

*cpm/10^6 cells/4 hours. Value given is mean ± SE for three separate determinations.

†cpm/10^6 cells/24 hours. Value given is mean ± SE for seven separate determinations.

‡P not significant.

§$P < 0.01$, Student's t test.

Table 2. Methionine Synthase Activity in Extracts of Cultured Patient's and Control Fibroblasts and Lymphoblasts

Cells	Specific Activity* (nmol/hour/mg protein) Total Enzyme	Holoenzyme
Fibroblasts†		
Control	2.6 ± 0.3	0.6 ± 0.1
Patient	0.8 ± 0.2‡	0.4 ± 0.1
Lymphoblasts		
Control	2.7 ± 0.2	0.9 ± 0.01
Mother	1.9 ± 0.2	0.8 ± 0.06
Father	2.5 ± 0.3	1.3 ± 0.04
Patient	0.8 ± 0.1‡	0.4 ± 0.03‡

*Values given are the mean ± SE, in excess of six separate determinations.

†Fibroblasts were cultured in Eagle's minimum essential medium supplemented with 10% FBS. Cells were harvested upon confluency and assayed for methionine synthase activity as described in the text. Lymphoblasts were cultured in RPMI 1640 medium supplemented with 10% FBS and cells harvested 48 hours after initiation of culture and assayed for methionine synthase activity.

‡Values significantly different from control levels at $P < 0.01$, Student's *t* test.

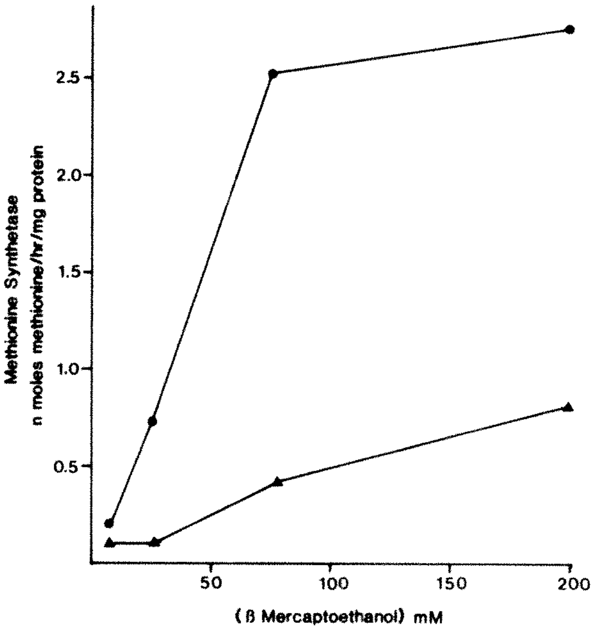

Fig 1. Levels of methionine synthase of patient and control lymphoblasts assayed with varying B mercaptoethanol concentrations. Lymphoblasts were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, cells were harvested and assayed for methionine synthase as described in Materials and Methods, with B mercaptoethanol concentration ranging from 7.5 to 200 mmol/L. The values given are the mean of duplicate determinations. ●, Control; ▲, patient.

without added cobalamin (Table 2). Both holoenzyme and total enzyme activities were reduced in patient's fibroblasts and lymphoblasts. This reduced methionine synthase activity was evident in patient's lymphoblasts in culture at all intervals up to 96 hours. Assay of methionine synthase of patient and control lymphoblasts under anaerobic conditions showed no significant difference to that under aerobic conditions. Methionine synthase of control lymphoblasts were 2.2 and 0.9 (holoenzyme) nmol/hour/mg protein (aerobic) and 2.3 and 0.6 (anaerobic). Corresponding values for patient lymphoblasts were 0.36 and 0.21 (holoenzyme) nmol/hour/mg protein (aerobic) and 0.40 and 0.30 nmol/hour/mg protein (anaerobic). In data not shown, mixing control and patient lymphoblast extracts yielded the expected levels of methionine synthase activity, excluding the possibility of inhibition or, alternatively, activation of patient's reduced methionine synthase activity by constituents of control extracts.

Effect of alteration of assay conditions on methionine synthase of patient's cells. Rosenblatt et al[4] have demonstrated that methionine synthase in the previously described patient with megaloblastic anemia and homocystinuria was normal at conventional thiol concentrations. The effect of varying assay concentrations of dithiothreitol (DTT 0.2 to 25 mmol/L) and B mercaptoethanol, (7.5 to 200 mmol/L) were determined with lymphoblast extracts of the current patient. In both instances at all concentrations of thiol assayed the patient's methionine synthase activities (Figs 1 and 2) were significantly lower than those of control lymphoblasts. For both patient and control lymphoblast extracts, methionine synthase activity at the maximal concentration of DTT (25 mmol/L) was approximately four times that observed with B mercaptoethanol (200 mmol/L), suggesting that the reducing conditions produced by these thiol reagents are neither equivalent nor a unique interaction of DTT with methionine synthase. Methionine synthase of patient and control fibroblasts were also assayed with varying concentrations of B mercaptoethanol: 5, 75 and 200 mmol/L. With these thiol concentrations, control fibroblast methionine synthase activities were 0.17, 1.45, and 2.5 nmol/hour/mg protein. Corresponding values for patient fibroblast methionine synthase were 0.04, 0.39, and 0.8 nmol/hour/mg protein. In data not shown, increasing the assay concentration of methylcobalamin (up to 50-fold), S-adenosyl methionine (up to fivefold), and 5 methylTHF (up to fivefold) did not significantly alter the reduced methionine synthase activity exhibited by patient cell extracts. In addition, substitution of methyl cobalamin by hydroxocobalamin or cyanocobalamin in the assay system produced no significant differences.

Effect of varying culture concentration of cobalamin on methionine synthase activity. The methionine synthase activities for control and patient lymphoblasts cultured with either varying cobalamin compounds or concentration are shown in Table 3. Control lymphoblasts cultured with increasing concentration of cyanocobalamin (0.5 μg/L, 0.375 nmol/L to 500 μg/L, 375 nmol/L) exhibited a fivefold increase in methionine synthase, and the enzyme levels with hydroxoCbl or cyanoCbl at the higher cobalamin culture concentration used were comparable (Table 3). Patient's lymphoblasts cultured under identical conditions showed a twofold increase in methionine synthase activity, and this activity level was now in the normal range.

Accumulation and distribution of labeled cobalamin and effect of methionine culture deprivation on cell growth. The accumulation of label from 57Co-cyanocobalamin in patient and control lymphoblasts after two hours or 24 hours

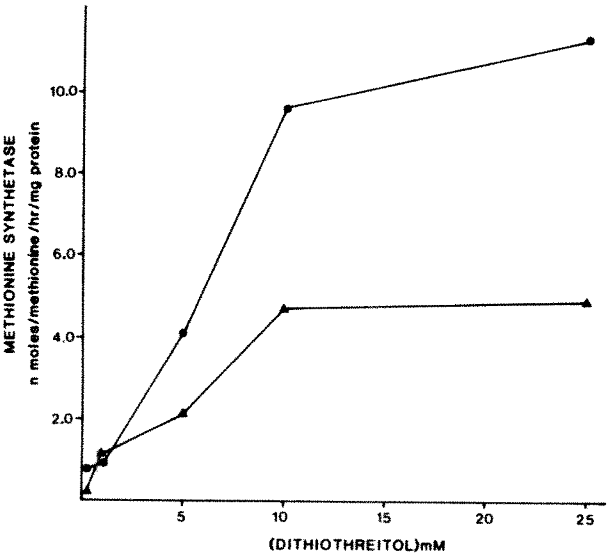

Fig 2. Levels of methionine synthase of patient and control lymphoblasts assayed with varying dithiotreitol concentrations. Lymphoblasts were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, and cells were harvested and assayed for methionine synthase as described in Materials and Methods, with dithiothreitol ranging from 0.2 to 25 mmol/L. The values given are the mean of duplicate determinations. ●, Control; ▲, patient.

in culture was comparable: at two hours patient 3.2×10^3 cpm, control 2.4×10^3 cpm/10^6 cells; at 24 hours patient 19×10^3 cpm, control 17×10^3 cpm/10^6 cells. The distribution of methylCbl and adenosylCbl in human lymphoblasts showed that in contrast for fibroblasts the major cellular cobalamin is adenosylCbl, but the concentration of methylCbl in patient's lymphoblasts and fibroblasts was markedly reduced (Table 4). The patient's cells exhibited reduced growth rates when cultured in methionine-replete medium or when methionine was replaced by homocysteine. When 2×10^5/mL control and patient fibroblasts were plated in complete medium, growth at 96 hours for control cells was $9 \pm 0.3 \times 10^5$ (SEM)/mL and for the patient's cells $5 \pm 0.2 \times 10^5$/mL. When homocysteine (0.2 mmol/L) was substituted for methionine in the media, growth at 96 hours was $8 \pm 0.5 \times 10^5$/mL for control fibroblasts and $3 \pm 0.2 \times 10^5$/mL for the patient fibroblasts. Lymphoblasts were also studied. Both control and patient lymphoblasts cultures were initiated at a cell density of 0.5×10^6 cells/mL. Growth at 96 hours in methionine replete medium was $3.2 \pm 0.3 \times 10^6$ (SEM)/mL for the control lymphoblasts and $2.4 \pm 0.1 \times 10^6$/mL for patient's lymphoblasts. Both the control and patient lymphoblasts showed a preferential growth requirement for methionine with the patient's lymphoblasts exhibiting no increase in cell numbers in the absence of methionine. When homocysteine was substituted for methionine, control lymphoblasts' density at 96 hours had increased to $0.9 \pm 0.1 \times 10^6$ (SEM)/mL, whereas the patient's lymphoblast density at 96 hours was $0.4 \pm 0.1 \times 10^6$/mL.

Table 3. Methionine Synthase Activity of Patient's and Control Lymphoblasts Cultered Under Varying Cobalamin Conditions

Culture Conditions*	Concentration of Added Cobalamin CyanoB12				HydroxoB12
	nmol/L				
	0.375	3.75	187	375	375
	Methionine synthase (nmol/hour/mg protein)				
Control	1.8	2.8 (0.8)‡	8.1	9.0 (1.3)	13.0
Patient	0.8	0.9 (0.4)	1.7	1.7 (0.7)	21

Culture conditions† MethylTHF	+ HydroxoB12	− HydroxoB12
	Methionine synthase (nmol/hr/mg protein)	
Control	4.8	2.1
Patient	1.5	0.6

*Lymphoblasts were cultured in standard RPMI 1640 supplemented with 10% fetal calf serum with varying concentrations of cyanocobalamin or hydroxocobalamin as indicated. Cells were harvested at 48 hours and assayed for methionine synthase activity as described in the text.

†Lymphoblasts were cultured in RPMI 1640 medium without or with hydroxocobalamin at concentration of 3.75 nmol/L and methylTHF 2μmol/L, harvested at 48 hours, and assayed for methionine synthase as described in the text.

‡Figures in parenthesis are holoenzyme values.

DISCUSSION

Inherited defects of cobalamin metabolism resulting in megaloblastic anemia and homocystinuria include the mutations of congenital methylmalonic aciduria-homocystinuria. On the basis of biochemical and genetic complementation studies, these disease states represent at least two different genetic entities termed CblC and CblD mutations, which both involve an assumed defect in Cob(III)alamin reductase.[14-17] This activity reduces the charge of the cobalt of cobalamin from +3 to +2, an event that normally occurs after cobalamin enters into the cell and precedes the formation of both adenosylCbl and methylCbl. Thus common to these mutations is the inability of affected cells to accumulate cobalamin as adenosylCbl and methylCbl and reduced holoenzyme activities of methylmalonylCoA mutase and methionine synthase.[1] Clinical findings include methylmalonic aciduria, homocystinuria, neurologic disorders, and a variable incidence of megaloblastic anemia confined to individuals with CblC mutation.[1] Cells of our patient (1) were not defective in accumulating cobalamin but exhibited decreased methyl Cbl but not adenosylCbl content, (2)

Table 4. Distribution of Methylcobalamin and Adenosylcobalamin in Lymphoblast and Fibroblast Extracts

Cell Line	SO3-B12	CN-B12	CH3-B12	Ado-B12	OH-B12
	% of Total Cobalamin*				
Lymphoblast					
Control	3	2	25	60	7
Patient	4	0	3	87	7
Fibroblast					
Control	3	0	58	31	8
Patient	3	0	10	76	11

*Results expressed as % of total cobalamin in cell extracts following growth for 72 hours in RPMI 1640 medium containing 57Co-cyanocobalamin (0.1 ng/mL). Values given are means of two separate determinations in duplicate.

utilized the methyl group of methylTHF less effectively than normal cells, and (3) incorporated the radio label from 14C propionate into protein at normal rates. In addition, our patient exhibited megaloblastic anemia with homocystinuria but not methylmalonic aciduria. These findings exclude CblC or CblD mutations and are identical with those reported for the patient with CblE disease. At variance are the differing characteristics of methionine synthase. Specifically, the reduced methionine synthase activities of our patient's lymphoblasts and fibroblasts were not normalized with optimal assay reducing conditions, either with DTT or B-mercaptoethanol. In addition, increasing substrate concentrations of methylTHF, methylCbl, or S-adenosylmethionine had no effect on the reduced activity. Although these findings suggest that our patient with altered methionine synthase activity may have a defect at the same locus as that of the previously described patient with congenital homocystinuria and abnormal methionine synthase activity,[3] only genetic complementation studies will resolve this question. That this altered methionine synthase is of significance for intact cells is suggested by the reduced methylCbl content of the patient's lymphoblasts and fibroblasts and an impaired growth rate of patient fibroblasts and lymphoblasts when methionine was substituted in culture medium by homocysteine. The mechanisms involved in methionine-dependent cell growth are controversial and evolving.[18,19] In the absence of extracellular methionine, this amino acid in cells is generated in human cells from either homocysteine by methionine synthase or by a series of reactions from the methylthio-containing portion of methylthioadenosine, a byproduct of polyamine metabolism.[20,21] Methionine for cell growth is potentially a summation of the contributions of these two pathways. The differing growth capacity of our patient's cells in culture when methionine has been replaced by homocysteine reflects in part the reduced methionine synthase activity of these cells and the relative contribution of this activity to the cellular methionine pool. Changing culture cobalamin conditions produced differing effects for the patient's and control lymphoblasts' methionine synthase activity. An increase in the concentration of cobalamin added to the culture medium was accompanied by a fivefold increase in this activity of control lymphoblasts compared with a twofold change for patient's cells cultured under identical conditions. Limiting cobalamin in the culture medium was accompanied by greater degrees of reduction of patient's activity, and it is of some interest that our patient's serum B12 on presentation was suboptimal. Although the magnitude of increase of patient's methionine synthase with increased cobalamin in the culture medium was less than that exhibited by control lymphoblasts, the absolute level of activity achieved by the patient was of the same order as in normal cells grown under standard conditions for cobalamin. This finding may have in vivo significance since both the homocystinuria and megaloblastic anemia responded completely to large doses of cyanoCbl, and it is feasible that this is mediated by augmentation of the reduced activity in vivo. The molecular basis for cobalamin-related changes in methionine synthase of culture cells is not entirely clear. It is independent of protein synthesis and probably reflects stabilization of enzyme by cobalamin with decreased enzyme turnover.[11] If this is so, the differing pattern of response of the patient's methionine synthase to cobalamin concentration in culture suggests a fundamental difference for mutant activity. Only purification and characterization of the enzyme will satisfy this hypothesis. As the two described patients with neonatal megaloblastic anemia, homocystinuria, and altered methionine synthase activity exhibited neurologic features, including seizures and developmental delay, the impact of altered cobalamin metabolism on the developing neurologic system deserves some comment. Whether in hereditary disorders such as transcobalamin II deficiency, CblC, and now CblE mutation or the acquired defects of vitamin B12 in breast-fed infants of vegan or pernicious anemia mothers,[5,22] the accompanying neurologic syndromes are characterized by their diversity if one sets aside the background of failure to thrive, listlessness, and irritability. Possibly the specific neurologic syndromes depend primarily on the developmental stage of the infant at the onset of vitamin deficiency. Whatever the manifestations, failure to diagnose the specific defect and to institute appropriate therapy can have dire consequences for the infant's ultimate neurologic status,[22-24] whereas early recognition is associated with a favorable outcome as illustrated in our patient.

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Correlation of Drug-Perturbed Marrow Cell Growth Kinetics and Intracellular 1-B-D-Arabinofuranosylcytosine Metabolism With Clinical Response in Adult Acute Myelogenous Leukemia

By Judith E. Karp, Ross C. Donehower, Gregory B. Dole, and Philip J. Burke

To define the relationship between leukemic cell growth, intracellular metabolism of 1-B-D-arabinofuranosylcytosine (ara-C), and the clinical response to timed sequential induction therapy with ara-C in adult acute myelogenous leukemia (AML), growth kinetic and biochemical pharmacologic determinants were examined in AML bone marrow populations. Leukemic blasts from 45 previously untreated patients obtained prior to therapy were cultured in vitro in autologous pretreatment serum (APS) and in serum containing drug-induced humoral stimulatory activity (HSA). Cell populations cultured in HSA demonstrated both increased proliferation, as measured by both [^{3}H]dThd incorporation into DNA and [^{3}H]dThd leukemic blast labeling index, and greater [^{3}H] ara-C leukemic blast labeling index relative to cells maintained in APS. HSA-cultured marrow cells from the 31 patients who achieved complete remission with ara-C-containing therapy demonstrated enhanced intracellular formation of ara-C 5′-triphosphate over three hours and retention of this active form during one subsequent hour in drug-free medium relative to cells maintained in APS. In contrast, cells from the 14 nonresponsive patients demonstrated no such HSA-induced increases in intracellular ara-C metabolism. These studies of human AML marrow cells identify behavior patterns of ara-C activation and net metabolism in the kinetically perturbed, proliferative state that may discriminate clinical sensitivity from clinical resistance to ara-C-based timed sequential therapy. Sensitive AML populations behave similarly to normal hematopoietic cohorts, with direct linkage of HSA-perturbed growth and pharmacologic parameters, while refractory cells demonstrate uncoupling of these determinants in the growth-stimulated state. These in vitro measurements may further serve as a template for prediction of clinical outcome to timed sequential therapy with ara-C, where both pharmacologic and cytokinetic determinants of response are intrinsic to the success of the designed drug scheduling.

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THE PHARMACOLOGIC basis for 1-B-D arabinofuranosylcytosine (ara-C) cytotoxicity rests on its intracellular conversion to the active metabolite, ara-C 5′-triphosphate (ara-CTP), which acts as a competitive inhibitor of DNA polymerase[1-3] and is directly incorporated into DNA, with further deleterious effects on DNA structure and function.[4-6] Therefore the extent to which ara-C is metabolized to ara-C 5′-triphosphate (ara-CTP) and the intracellular distribution of this active metabolite in blast cells prior to treatment have been examined in vitro as possible predictors of clinical drug sensitivity for acute myelogenous leukemia (AML) cell populations.[7-14] Such studies have not consistently correlated closely with clinical outcome, suggesting that measurement of an isolated determinant of ara-C activity is not sufficient to discriminate responders from nonresponders. The inability of these single-parameter studies to predict response may relate not only to biological patient-to-patient heterogeneity, which limits the applicability of specific assay systems,[15-18] but perhaps also to cellular heterogeneity within an individual tumor population that may obscure detection of drug-resistant cell subpopulations.[19-22]

One factor that may have major impact on overall ara-C cytotoxicity is the growth characteristics of the target cell population. ara-C is a cell cycle-dependent antimetabolite with its greatest toxicity directed against cells in S phase.[23] As such, the overall mechanism of action and net toxic effects should depend on the fraction of proliferating cells in the treated population at the time of drug exposure. In this light, growth kinetic heterogeneity, a characteristic of pretreatment leukemic cell populations,[21,24-30] may in part account for the apparent failure of measurements of intracellular ara-C biochemical pharmacology alone to accurately predict net drug effect and clinical response.

The development of timed sequential therapy for adult AML at the Johns Hopkins Oncology Center is based on the study of drug-primed cell growth kinetics in several systems[25,26,29,31-37] and is designed to overcome pretreatment growth heterogeneity by recruitment of residual leukemic cells into DNA synthesis following initial cytoreduction. Our studies have shown that after an initial dose of cytotoxic therapy, various measures of cell proliferation reach a maximum at a predictable time following the initial drug(s).[26,29,32,33,35,37] This growth perturbation in both man and rodent is associated with induction of serum activities that promote bone marrow cell DNA synthesis in vitro.[26,31,38-41] This maximally detected humoral stimulatory activity (HSA) also occurs at a predictable time following initial aplasia-producing drug administration and coincides with the increased [^{3}H]dThd labeling index (LI) and clonogenicity of recovering normal granulocytic and malignant hematopoietic precursors in vivo.[24,26,29,32,33,35,38,41] The drug-induced kinetic homogeneity and its relation to improved antitumor effects of ara-C have been demonstrated in vivo in leukemia-bearing rodents, where the detection of HSA and peak drug-induced tumor LI have been correlated with enhanced therapeutic effects of ara-C given in a sequence based on this predictable increase in residual tumor proliferation.[24,26,34,39]

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To define the relationship between perturbed cell growth and intracellular ara-C metabolism in human cells, we examined growth kinetic and ara-C biochemical pharmacologic determinants in HSA-perturbed normal human bone marrow populations.[42] Those studies demonstrated the coupling of induced cell growth with enhanced ara-C activation and metabolism in the kinetically perturbed, proliferative state. In our present studies we have now examined growth kinetic and biochemical pharmacologic determinants in pretreatment human AML bone marrow cell populations in vitro, both in the baseline milieu of autologous pretreatment serum (APS) and in the kinetically perturbed state in the presence of drug-induced HSA, and have been able to identify patterns in these in vitro studies that appear to correlate with clinical sensitivity and clinical resistance to ara-C-based timed sequential therapy.

MATERIALS AND METHODS

Patients and therapy. From April 1984 through January 1986, a total of 59 newly diagnosed, nonpreviously treated (NPT) adults (median age 54 years, range 19 to 76) with the diagnosis of AML (myeloid, myelomonocytic, monocytic, megakaryocytic) were consecutively admitted to the Adult Leukemia Service of the Johns Hopkins Oncology Center for intensive timed sequential induction chemotherapy with ara-C and daunorubicin[35,36] and were evaluated with respect to clinical outcome following treatment. All patients received antileukemic therapy as part of clinical investigation approved by the Joint Committee on Clinical Investigation of the Johns Hopkins University School of Medicine in accord with assurance approved by the US Department of Health and Human Services, and following full informed consent to participate in active investigative therapy. Of these 59 NPT patients, a total of 45 (76.3%) were studied and were considered able to be evaluated with respect to the interrelationship of growth, pharmacologic parameters, and clinical response. Five (8.5%) of the 59 patients did not survive induction chemotherapy and were therefore not considered able to be evaluated for response.[35,36] An additional nine (15.3%) patients did not have pretreatment studies performed; three of these patients had severe myelofibrosis and the remaining six required emergency institution of cytoreductive therapy for leukostasis. Of the 59 consecutively admitted patients, 38 (64.4%) achieved complete remission (CR) while 16 (27.1%) had no response (NR) to induction therapy. Of the 45 patients studied and evaluated for in vitro growth and ara-C pharmacology plus clinical outcome, all had at least 80% malignant marrow blasts prior to treatment. CR of at least 3-months duration, defined by Acute Leukemia Group B criteria, was achieved following induction therapy in 31 (69%) of these NPT adults; the remaining 14 patients had NR to timed sequential therapy (or CR less than 3 months).

Bone marrow cell cultures. Marrow cells were obtained from all 45 patients prior to therapy by routine needle aspiration, collected in RPMI 1640. To ensure that marrow cells were free from contamination by peripheral blood blasts, marrow spicules were isolated, and cells isolated from the spicules were dispersed as single cells by drawing through a 25-gauge needle. Each cell suspension was prepared and studied individually. Whole marrow cell suspensions (1×10^6 cells/mL for incorporation and labeling index studies; 2.5×10^6 cells/mL for ara-CTP formation and retention studies) were cultured in replicate in sterile plastic Falcon 2063 and 2057 tubes with specific sera at 15% concentration. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Specific sera collection. Sera were obtained from all newly diagnosed AML patients. Prior to treatment, 100 mL of whole blood were collected from each patient and allowed to clot and the serum was separated under sterile conditions and stored immediately at −70°C. These sera were studied in culture with bone marrow cells from the same patient (autologous pretreatment sera, APS).

Drug-induced HSA-containing sera were obtained from patients with AML undergoing aplasia-inducing chemotherapy at the predicted peak of maximal detection.[31-33,35,38,40,43] To obtain adequate amounts of HSA for these in vitro studies, post-treatment sera from ten leukemia patients achieving CR, which maximally stimulated in vitro marrow cell [^{3}H]dThd incorporation relative to the effects of normal serum, were pooled.[32,33,42,43] All sera were stored at −70°C until use.

[^{3}H]dThd incorporation assay. The method for assaying [^{3}H]dThd incorporation into marrow cell DNA has been described previously[31,38,40-43] and determines both the proliferative status of those marrow cells and the relative growth kinetic changes induced by perturbing the initial pretreatment cell population with HSA during 18 hours culture. In brief, marrow cell suspensions (1×10^6 cells/mL) were incubated with 15% specific serum plus [^{3}H]dThd 1 μC/mL (methyl-^{3}H, New England Nuclear, Boston; specific activity 2 Ci/mmol) at culture initiation. Following 18 hours incubation, triplicate cultures were terminated by immersion in ice, and the contents of each tube was collected under vacuum on glass fiber filter. Each tube was washed with 0.9% NaCl solution, the acid-insoluble materials on the filter were precipitated with cold 5% trichloracetic acid, and the precipitate was washed three times with absolute ethanol. The dry filter was transferred to a screw-top vial to which 6 ml of scintillation fluid (Instagel) were added, and the amount of incorporated isotope was counted in a liquid scintillation spectrometer. Results are expressed as pmol dThd incorporated/10^6 cells in specific sera.

Labeling index (LI) determinations. [^{3}H] ara-C and [^{3}H]dThd leukemic blast LI were determined following 18 hours of culture in APS and HSA, as described previously.[25,26,29,33,37,38,42] In brief, aliquots of monodispersed AML marrow were incubated with 15% specific serum and either [^{3}H] ara-C 1 μCi/mL (specific activity 11.2 Ci/mmol) or [^{3}H]dThd 0.1 μCi/mL (specific activity 2 Ci/mmol) for 75 minutes at 37°C in a humidified atmosphere containing 5% CO_2. Following incubation cells were washed carefully in phosphate-buffered saline (PBS) three times and lightly cytocentrifuged (1,000 × g, five minutes) onto slides coated with gelatin. Autographs were prepared with Kodak NTB-2 photographic emulsion, exposed for 21 days, developed, and stained with Giemsa. The [^{3}H] ara-C and [^{3}H]dThd LI of leukemic marrow cells were determined by counting the number of cells per 1,000 that contain >5 grains overlying the nucleus. Background labeling is estimated by the number of grains present in a cell-free area equivalent to the area of the leukemic blast nucleus. Background labeling is negligible for [^{3}H]dThd (≤ 1 grain) and is ≤ 2 to 3 grains for [^{3}H] ara-C. Cells labeled with [^{3}H] ara-C generally contain ≥ 10 grains overlying the nucleus, and cells labeled with [^{3}H]dThd generally contain ≥ 25 grains. Results are expressed as percentage of labeled blasts. The standard error for this method is ±1% in our laboratory.

Intracellular ara-CTP levels. [^{3}H] ara-C (5,6-^{3}H cytosine; Moravec Biochemicals, City of Industry, CA; specific activity 18 Ci/mmol) was added to duplicate cultures at time 0 and to cells in APS and HSA following 18 hours incubation (Time 18) to yield a final ara-C concentration of 1 μmol/L. Because ara-C uptake is linear over one hour and intracellular ara-CTP formation with subsequent retention continues with a plateau over the following one to two hours,[8-10] cultures were incubated at 37°C in humidified 5% CO_2 for three hours, then centrifuged at 5,000 × g for five minutes, and the ara-C-containing medium was decanted. Half of the cultures were resuspended in fresh medium without drug and were returned to the incubator to be analyzed one hour later for ara-CTP retained

Table 1. Comparison of Effect of Autologous Pretreatment Serum (APS) and Humoral Stimulatory Activity (HSA) on Measurements of DNA Synthesis

	APS	HSA	Δ*	P†
$[^3H]$dThd Incorporation (pmol/10^6 cells)				
CR‡	10.1§ (2.5-34.6)‖	14.0 (2.8-49.5)	1.37 (0.77-2.62)	<0.0003
NR	13.1 (3.2-26.0)	18.9 (5.7-34.5)	1.52 (0.96-2.28)	<0.003
$[^3H]$dThd LI (% labeled leukemic blasts)				
CR	6.3 (0.8-14.1)	8.9 (1.0-18.4)	1.56 (0.55-3.00)	<0.005
NR	6.0 (2.6-11.6)	9.1 (2.7-12.1)	1.54 (1.04-3.47)	<0.009
$[^3H]$ ara-C Incorporation (pmol/10^6 cells)				
CR	1.8 (0.2-4.7)	2.0 (0.3-4.2)	1.19 (0.48-2.87)	0.12
NR	1.5 (0.4-2.8)	1.9 (0.7-4.6)	1.26 (0.65-3.85)	0.21
$[^3H]$ ara-C LI (% labeled leukemic blasts)				
CR	5.4 (0.4-14.5)	8.5 (0.7-18.3)	2.05 (0.85-12.5)	<0.0001
NR	6.5 (2.4-12.6)	9.0 (2.6-17.5)	1.37 (0.79-2.48)	<0.02

*Δ HSA/APS; change induced by culture in HSA relative to maintenance in APS.

†Paired two-tailed *t* test (values in HSA *v* values in APS).

‡CR, complete remission, 31 patients; NR, no response, 14 patients.

§Mean for all CR or NR marrow populations.

‖Range for individual CR or NR marrows.

following the end of drug exposure in the manner described below. The other half of the cultures were subjected to a 0.8 mol/L perchloric acid precipitation. Following centrifugation the supernatant liquid containing the acid-soluble cell constituents was transferred to another tube where it was neutralized with KOH to a pH of approximately 7. The insoluble salt was removed by centrifugation, and the second supernate was immediately ready for high performance liquid chromatographic (HPLC) analysis of ara-C nucleotide levels. ara-CTP levels were measured on a Waters HPLC system with an M-45 pump, U6K injector, and model-440 variable wavelength absorbance detector set at 254 nm to monitor the separation (Waters Association, Milford, MA). The isocratic separation used 0.4 mol/L KH_2 PO_4, pH 3.1, over a Whatman Partisil 10-SAX anion exchange column (Whatman, Clifton, NJ) with an elution time of six minutes and a flow rate of 3 mL/min. ara-CTP levels were quantitated by the recovery of ara-C-associated counts at the established retention time for ara-CTP and were expressed as pmol nucleotide/10^6 cells. Coefficient of variation for replicate samples is $\leq 10\%$ in our laboratory. This method is similar to those widely used for chromatographic separation of nucleotides.[10-12,44] The efficiency of the extraction of nucleotide triphosphates using this method was examined by adding a known quantity of radiolabeled triphosphate (deoxycytidine 5′-triphosphate [5-^{3}H], 15 Ci/mmol; New England Nuclear, Boston) to an untreated cell preparation. The extract was then analyzed by HPLC as described. Using this method 87.0% ± 6.0% of the standard remained in triphosphate form.

Statistical methods. Comparisons of the effects of HSA relative to the effects of APS (Δ HSA/APS) on leukemic marrow cell proliferation and intracellular ara-C metabolism were analyzed using Student's *t* test and Fisher's exact *t* test. The relationships between HSA-induced changes in growth and pharmacologic parameters and clinical outcome (CR, NR) were also analyzed using Student's *t* test and Fisher's exact *t* test.

RESULTS

Measurements of DNA synthesis. Measurements of cell growth kinetics by $[^3H]$dThd incorporation assay and LI of

Table 2. Comparisons of Effect of Autologous Pretreatment Serum (APS) and Humoral Stimulatory Activity (HSA) on Measurements of Intracellular ara-C Pharmacology Relative to Clinical Outcome

	APS	HSA	Δ*	P†
Intracellular ara-CTP Formation (pmol/10^6 cells)				
CR‡	1.9§ (0.2-7.6)‖	2.7 (0.17-11.0)	1.53 (0.50-4.50)	<0.006
NR	2.2 (0.6-5.6)	2.4 (0.80-5.0)	1.29 (0.57-4.20)	0.09
Intracellular ara-CTP Retention (pmol/10^6 cells)				
CR	1.2 (0.1-4.8)	2.1 (0.15-8.6)	2.00 (0.41-5.19)	<0.002
NR	1.2 (0.3-3.6)	1.3 (0.30-5.8)	1.18 (0.63-4.83)	0.52
Relative Retention (%)¶				
CR	70.2 (11-210)	84.0 (22-281)	1.42 (0.61-4.25)	<0.03
NR	59.4 (25-120)	51.8 (25-140)	0.85 (0.35-1.30)	0.20

*Δ HSA/APS; change induced by culture in HSA relative to maintenance in APS.

†Paired two-tailed *t*-test (values in HSA *v* values in APS).

‡CR, complete remission, 31 patients; NR, no response, 14 patients.

§Mean for all Cr or NR marrow populations.

‖Range for individual CR or NR marrows.

¶Intracellular ara-CTP retention/intracellular ara-CTP formation (R/F).

the individual AML marrow cell suspensions from the 45 NPT patients demonstrated similar and reproducible behavior among this population, regardless of clinical outcome (Table 1). The HSA-induced increase in cell proliferation was similar to that detected for normal hematopoietic populations in vitro[42] and was similar for the 31 CR and 14 NR patients. Significant increases in both parameters were detected for AML cells from both CR and NR patients cultured in HSA relative to cells maintained in the pretreatment milieu of APS by 18 hours of culture. The incorporation of $[^3H]$ ara-C into DNA by these AML populations demonstrated greater variability than the counterpart $[^3H]$dThd assay, similar to our findings for normal hematopoietic populations,[42] without consistently significant effects of HSA detected for all patients. However, the effect of HSA on the $[^3H]$ ara-C LI of cultured AML populations was similar to that noted for $[^3H]$dThd incorporation and LI, with significant increases detected in $[^3H]$ ara-C leukemic blast LI.

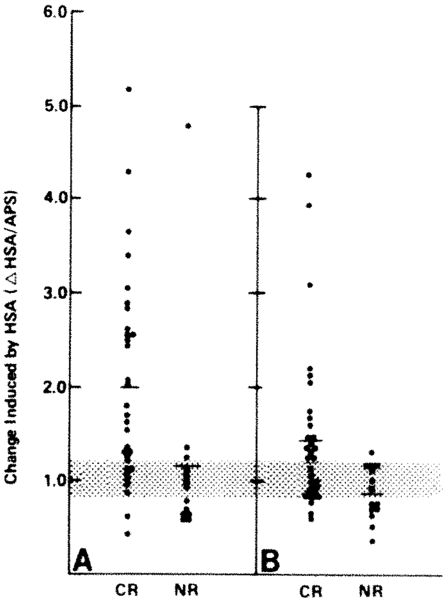

Fig 1. Effect of HSA on measurements of intracellular Ara-CTP retention relative to clinical outcome. The change induced by HSA relative to the effect of APS in (A) quantitative intracellular Ara-CTP retention (Δ R) and (B) relative retention (Δ R/F) is depicted for each of the individual 31 CR and 14 NR patients. Mean Δ R and Δ R/F for the CR and NR subgroups are depicted by horizontal bars. The shaded area represents Δ of 0.80 to 1.20; nonshaded areas represent significant induced changes for individual marrow populations.

Intracellular ara-C metabolism. The HSA-induced alterations in intracellular ara-C metabolism by cultured AML marrow cells demonstrated markedly different patterns for CR and NR patients (Tables 2 and 3, Fig 1). These differences, however, were detected only after an 18-hour exposure to HSA, when peak measured proliferation is detected.[38-43] Determinations of three-hour intracellular ara-CTP formation at the time of culture initiation (time 0) were similar for all marrow cell populations cultured in APS (CR, mean 6.1 pmol ara-CTP/10^6 cells, range 0.9 to 27; NR, mean 7.1 pmol/10^6 cells, range 1.1 to 24) and those cultured in HSA (CR, mean 7.6 pmol/10^6 cells, range 1.0 to 30; NR, mean 7.7 pmol/10^6 cells, range 1.0 to 22). Thus, similar to the findings of others,[8,9,12] we did not detect significant differences in baseline AML intracellular ara-C metabolism between CR and NR patients. Likewise, measurements of subsequent 1-hour intracellular ara-CTP retention and relative percentage of ara-CTP retained (R/F) in APS-cultured cells were similar for CR (mean retention 4.8 pmol/ 10^6 cells, range 0.6 to 19; mean R/F 70.5%, range 29 to 112) and NR (mean retention 4.3 pmol/10^6 cells, range 0.5 to 14; mean R/F 69%, range 36 to 101) subgroups. Cells from CR patients that were exposed to HSA during the initial three hours of culture tended to have greater intracellular ara-CTP retention (mean 5.8 pmol/10^6 cells, range 0.8 to 20; mean Δ HSA/APS 1.37, range 0.62 to 3.62, $P = 0.09$) and R/F (mean 82.6%, range 10 to 162; mean Δ HSA/APS 1.14, range 0.34 to 2.25, $P = 0.12$) than APS-cultured cells, while

Table 3. Relationship of HSA-Induced Change in Intracellular ara-C Metabolism In Vitro to Clinical Outcome

	Clinical Outcome*		
	CR	NR	P†
Linked Growth and ara-C Metabolism‡			
Δ§ Formation ≥ 1.2	19/31 (61%)	6/14 (43%)	0.20
Δ Retention ≥ 1.2	22/31 (71%)	3/14 (21%)	<0.003
Parallel HSA-Induced Change in Growth and ara-C Metabolism	25/31 (81%)	2/14 (14%)	<0.00005
Uncoupled Growth and ara-C Metabolism			
Δ Formation ≤ 0.8	2/31 (6%)	4/14 (29%)	0.065
Δ Retention ≤ 0.8	2/31 (6%)	6/14 (43%)	<0.007
Divergent HSA-Induced Change in Growth and ara-C Metabolism	3/31 (10%)	10/14 (71%)	<0.00007

*CR, complete remission, 31 patients; NR, no response, 14 patients.
†Fisher's exact *t* test (CR *v* NR).
‡Intracellular ara-CTP levels.
§Δ HSA/APS, change induced by culture in HSA relative to maintenance in APS.

cells from NR patients demonstrated no such HSA-related trends in either retention (mean 4.9 pmol/10^6 cells, range 0.2 to 14) or R/F (mean 66.2%, range 10 to 90). Initial exposure to HSA resulted in modest increments in these pharmacologic measurements, particularly with respect to active drug retention by CR populations, but these HSA-related changes were not markedly different either from CR cells maintained in APS or from NR populations assayed during the initial phases of the culture period.

Following 18 hours in culture, however, the pharmacologic behavior of HSA-stimulated cells from CR patients was distinct from similarly cultured cells obtained from NR patients (Table 2, Fig 1). Significant increases in both three-hour intracellular ara-CTP formation ($P < 0.006$) and subsequent one-hour ara-CTP retention ($P < 0.002$) were detected for the 31 CR cell populations cultured in HSA relative to the same cells maintained in APS, with a greater HSA-related increase detected for retention than for formation ($P < 0.02$). In contrast, only modest HSA-induced enhancement of ara-CTP formation and no such enhancement of active drug retention was detected for cells from the 14 NR patients. Furthermore, although there was no significant difference in the absolute amounts of ara-CTP formed for CR and NR populations, the HSA-induced increase in ara-CTP retention by CR cells ($P < 0.01$) was significantly greater than that detected for NR cells. Parallel HSA-induced perturbations in growth kinetics and intracellular ara-C metabolism were associated with achievement of CR; conversely, divergence of cellular growth and pharmacologic behavior in the HSA-stimulated state was associated with clinical ara-C refractoriness (Table 3). The percent of relative intracellular ara-CTP retention (R/F) for cells from the 31 CR and 14 NR patients was similar when cells were maintained in APS for 18 hours and was similar to the same parameters obtained by immediate (time 0) exposure to ara-C in vitro. For the HSA-cultured CR populations, however, relative retention was magnified (Δ R/F, comparing R/F in HSA milieu with R/F in APS), unlike NR populations, with the percentage of ara-CTP retained by HSA-perturbed CR cells being greater than the percentage retained by HSA-cultured NR cells ($P < 0.01$).

DISCUSSION

These studies of human AML marrow cells examine the interrelationship between cell growth kinetics and intracellular biochemical pharmacology of the S-phase-specific antimetabolite ara-C. Measurements of these parameters are feasible and can be evaluated in at least 75% of our adult AML patient population. From our data we are able to identify behavior patterns of ara-C activation and net metabolism in the kinetically perturbed, proliferative state that may discriminate clinical sensitivity from clinical resistance to ara-C-based timed sequential therapy. The linkage of HSA-perturbed growth with HSA-enhanced intracellular drug disposition in clinically sensitive AML cohorts parallels our findings in normal human hematopoietic cells,[42] while clinically resistant leukemic populations demonstrate an uncoupling of proliferative and biochemical determinants in the growth-stimulated milieu. Such uncoupling likely signifies divergence from normal drug-sensitive behavior and, thus, resistance to ara-C cytotoxicity. These findings further provide a biochemical rationale to our observations regarding enhanced ara-C cytotoxic effects in HSA-stimulated populations and substantiate data derived from in vitro chemotherapy models in human hematopoietic cancers[33,43] as well as support our in vivo trials of timed sequential therapy in leukemia-bearing rodents[24,26,34,39] and in humans with acute leukemia.[31,35,36]

The in vitro effects of HSA were not immediately measurable either in terms of growth perturbation or enhanced net drug metabolism. This lag time with respect to both factors is not surprising given the mechanisms of action defined for the multiple hematopoietic stimulators.[39-41,45-47] HSA likely acts during G_1 and effects recruitment of target cells into S phase[24,32,39,41-43,45,46]; hypothetically its initial actions may relate to changes at the cell membrane level that affect nucleoside receptor number or binding and/or to induction of enzymes that promote intracellular nucleoside metabolism, ie, cytidine kinase, as well as enzymes such as thymidylate synthetase that facilitate DNA synthesis and eventually culminate in proliferation as measured by entry into S phase. The modest increments in pharmacologic parameters noted during the initial three to four hours of culture in HSA may reflect these postulated initial actions. The full effects of HSA perturbation, however, are realized only after an exposure time that allows for entry of those stimulated cell cohorts into S phase with ongoing DNA synthesis.

Our data demonstrate a differential effect of HSA on biochemical measurements of net intracellular ara-C metabolism by cultured AML populations with respect to eventual clinical outcome of ara-C-based timed sequential therapy. Although all HSA-perturbed populations evinced some increase in intracellular ara-CTP formation relative to APS-maintained cells, this enhancement tended to be greater in sensitive cohorts than in resistant cells. This enhancement may relate to changes induced at multiple potential sites where ara-C activity could be modulated, including membrane receptors and transport systems,[48] intracellular pool sizes of cytidine metabolites,[49,50] or enzymes responsible for ara-C activation.[4,14,51,52] However, the overall effect of HSA on this initial stage of intracellular ara-C metabolism does not clearly differentiate drug-sensitive from drug-resistant leukemic populations, suggesting that the underlying ara-C activation mechanisms affected by HSA are perhaps directly linked to mechanisms related to proliferation in both subgroups.

Determinations of quantitative and relative intracellular ara-CTP retention, as the measurement of net drug metabolism, in the growth-stimulated state appear to directly relate to eventual clinical outcome of ara-C-based timed sequential therapy. The HSA-related increase in activated ara-C retention measured in CR patients may well reflect an increased stability of triphosphorylated drug intracellularly. The paradoxical decrease in ara-CTP retention detected in growth-stimulated NR populations is less readily explained but may relate to increased levels of degradative enzymes or to efflux of active drug from the cell. Such potential defects cannot be determined from our data but are worth further consideration.

These data may further suggest a difference in both baseline and HSA-perturbed proliferation between CR and NR cell cohorts that could impact on pharmacologic behavior. Although all AML populations responded to HSA by increasing the proportion of cells in S phase and undergoing DNA synthesis, cells from NR patients tended to have modestly increased $[^3H]$dThd incorporation relative to cells from CR patients. The tendency for therapy-resistant cells from various hematologic malignancies to have higher initial DNA content and/or greater proportions of cells in apparent S phase has been detected by some investigators.[30,53,54] It is conceivable that some of our refractory AML cohorts may have an intrinsically shortened G_1 phase of the cell cycle that is further aggravated by exposure to HSA and during which the requisite processes leading to net drug activation, metabolism, and eventual incorporation into DNA cannot be carried to completion.

From our present studies we conclude that sensitive AML marrow cell populations behave similarly to normal hematopoietic cells[42] with direct linkage of growth and pharmacologic behavior that culminates in enhanced ara-C cytotoxicity in the HSA-perturbed nonsteady state, while refractory cells demonstrate uncoupling of these determinants. This in vitro model using HSA to partially overcome kinetic heterogeneity parallels timed sequential therapy, where initial drug priming induces proliferation of the residual tumor cohort in vivo[26,29,33,37,38] and supports the premise that such induced proliferation renders target cells more sensitive to the effects of an optimally timed ara-C infusion when those cells are capable of appropriate pharmacologic response. These in vitro measurements of interrelated growth and pharmacology may further serve as a template for prediction of clinical outcome to timed sequential therapy with ara-C, where both determinants of response are intrinsic to the designed drug scheduling.[24,29,31,35-37,39]

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The Molecular Basis of β-Thalassemia in Lebanon: Application to Prenatal Diagnosis

By F.F. Chehab, V. Der Kaloustian, F.P. Khouri, S.S. Deeb, and Y.W. Kan

A study of the molecular lesions of β-thalassemia in Lebanon revealed the presence of eight different mutations in 25 patients with Cooley's anemia. The IVS1 position 110 mutation predominated with a frequency of 62% and was almost invariably associated with Mediterranean chromosome haplotype I. Five other mutations commonly found in the Mediterranean area occurred with frequencies of 2% to 8%. In addition a G → C substitution in IVS1 position 5 (a lesion previously found in Chinese and Asian Indians) was demonstrated in a patient with Mediterranean haplotype IX. A new mutation at codon 29 was found in two other patients with haplotype II. The characterization of these β-thalassemia mutations should allow the implementation of a prenatal diagnosis program in that country.

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LEBANON, a small country on the Mediterranean Sea, is known to have interacted with many civilizations over the centuries, and the Lebanese population has resulted from an admixture of various ethnic groups. The incidence of β-thalassemia trait of both the β^0 and β^+ forms[1] is 2% to 3% in Lebanon.[2] Consanguineous marriages within certain ethnic communities having a high incidence of β-thalassemia have led to an increase in homozygous patients within that particular community. Clinically, homozygous β-thalassemia in Lebanon is manifested as the severe transfusion-dependent state, a form of Cooley's anemia that prevails in the Mediterranean region, throughout the Middle East, the Indian subcontinent, Burma, and southeast Asia.[3] The elucidation of the molecular defects of β-thalassemia in Lebanon should be informative on both scientific as well as anthropologic levels, since it would reflect the diversity of populations residing in that country. Whereas therapy for β-thalassemia has been mainly supportive, prevention of the disease by prenatal diagnosis has been a major goal in areas where the incidence is high. The characterization of the molecular defects would allow implementation of a prenatal diagnosis program. In this study, we have delineated the molecular lesions of β-thalassemia in Lebanon. We first studied the linkage of DNA polymorphisms in the β-globin gene cluster to specific β-thalassemia mutations and then defined specific mutations with oligonucleotide probes or by cloning and sequencing. We found eight different DNA haplotypes and eight different mutations among 50 β-thalassemia chromosomes. Six mutations were previously described in the Mediterranean population; one mutation previously found in the Chinese and Asian Indian populations was also found in this Mediterranean population on a new haplotype. In addition, we uncovered a new mutation that has not been previously described.

METHODS

Patients. Twenty-five unrelated homozygous β-thalassemic subjects (three β^0 and 22 β^+) were studied. All but one Christian patient were either Sunni or Shiite Moslems. Individuals in the present study have typical histories of transfusion-dependent Cooley's anemia.

DNA extraction and Southern blotting. DNA was extracted from the peripheral blood of affected individuals by either the proteinase K method[4] or by the standard procedure of phenol-chloroform extraction.[5] Ten micrograms of DNA were digested to completion with the appropriate restriction enzyme, separated according to size on agarose gels, transferred to nitrocellulose filters,[6] and hybridized to ^{32}P-nick translated β, γ, ψβ, and ε globin gene probes. DNA haplotypes were determined using seven common restriction site polymorphisms in the β-globin cluster.[7]

Oligonucleotide hybridization. Synthetic 19-mer oligonucleotide probes were labeled by phosphorylation of the 5′ ends with γ-^{32}P-ATP (7,000 Ci/mmol, ICN or New England Nuclear, Boston) and T4 polynucleotide kinase. The labeled oligonucleotide was then separated from unincorporated nucleotides by thin layer chromatography on diethyl aminoethyl (DEAE) cellulose plates (Analtech, Newark, DE) in the presence of urea and hydrolysed yeast tRNA.[8] Five to ten micrograms of genomic DNA were digested with Bam HI, separated on 0.8% agarose gels, and processed exactly as described,[9] except that the hybridization and washing temperatures were varied according to the probe used as shown in Table I. These temperatures were empirically selected according to the formula of Conner et al,[9] then experimentally adjusted.

Molecular cloning and DNA sequencing. One hundred fifty to two hundred micrograms of genomic DNA were completely digested with Eco RI or Hind III, fractionated on 10% to 40% sucrose gradients, and the β-globin-rich fraction identified by Southern blotting. Bacteriophage λ Charon 4A and Charon 30 were used for Eco RI and Hind III vectors, respectively. The recombinant bacteriophage clones containing β-globin inserts were generated as described.[10] Bacteriophage clones containing the 5.2 kb, 3.6 kb Eco RI fragments and 7.8 kb Hind III β-globin fragments were thus isolated. The 5.2 kb and 7.8 kb fragments were further subcloned in either of the bacterial plasmids pBR322, pUC18, or pBS (Stratagene Cloning Systems, San Diego). DNA sequencing of double-stranded supercoil DNA was essentially as described.[11]

Prenatal diagnosis. DNA from a Lebanese couple with a child affected by homozygous β-thalassemia was haplotyped for DNA polymorphisms in the β-globin cluster, and the most likely mutation

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Table 1. Hybridization and Washing Temperatures of Oligonucleotide Probes

Oligo	β-Globin Site		Sequence	Hybr/Wash
1	IVS1,110	Normal	5′ AAAATAGACCAATAGGCAG3′	54°/54°
		Mutant	5′ CTGCCTATTAGTCTATTTT 3′	45°/45°
2	IVS1,6	Normal	5′ AACCTTGATACCAACCTGC 3′	45°/58°
		Mutant	5′ GCAGGTTGGCATCAAGGTT 3′	48°/54°
3	Codon 39	Normal	5′ CCTTGGACCCAGAGGTTCT 3′	55°/55°
		Mutant	5′ AGAACCTCTAGGTCCAAGG 3′	55°/55°
4	Codon 8	Normal	5′ CTCCTGAGGAGAAGTCTGC 3′	45°/55°
5	Codon 29	Normal	5′ GGCCCTGGGCAGGTTGGTA 3′	52°/58°
		Mutant	5′ TACCAACCTACCCAGGGCC 3′	52°/55°
6	IVS1,5	Normal	5′ ACCTTGATACCAACCTGCC 3′	55°/53°
		Mutant	5′ GGCAGGTTGCTATCAAGGT 3′	55°/53°

linked to this haplotype was identified by oligonucleotide hybridization. After obtaining informed consent from the parents, 35 mL of amniotic fluid was collected in Lebanon and flown to San Francisco within 24 hours. The packed cells were collected by centrifugation, washed, resuspended in 400 μL of 0.1 mol/L NaCl, 0.05 mol/L Tris HCl pH 7.5, 1 mmol/L EDTA, and lysed with 25 μL of 10% SDS. The lysate was digested overnight with 100 μg/mL proteinase K at 55°C, phenol was extracted, and ethanol was precipitated. Five micrograms of DNA were digested with Bam HI and were processed as described above for oligonucleotide hybridization.

RESULTS

Haplotypes. One Asian and seven common Mediterranean haplotypes were found in the Lebanese population. Table 2 lists the haplotypes detected and their frequencies. Mediterranean haplotype I predominated over the other haplotypes. Twenty-three of the 25 patients were homozygous for a single haplotype (I, II, III, IV, VI, VII, and IX) and two were doubly heterozygous for two different haplotypes (I/VI and II/Asian H).

Detection of mutations. To elucidate the molecular defects of β-thalassemia in this population, we tested each patient's DNA by either Southern blotting or oligonucleotide hybridization for one or more mutations previously shown to be linked to that haplotype. If the molecular lesions could not be uncovered according to the above strategy, the β-globin genes were cloned and sequenced.

Two mutations were detected by Southern blotting. Patients with haplotype III and haplotype VII were tested for the IVS2 position 1 (G → T) and IVS2 position 745 (C → G) lesions that respectively alter the Hph I and Rsa I restriction endonuclease sites. One patient each was found to be homozygous for these two mutations.

Synthetic oligonucleotide hybridization detected the mutation in 18 other patients who are homozygous for a single haplotype. These include the IVS1 position 110 mutation in 15 β-thalassemia patients homozygous for haplotype I, the IVS1 position 6 mutation in a patient homozygous for haplotype VI, the nonsense mutation at codon 39 in a homozygote for haplotype II, and the dinucleotide deletion at codon 8 in a homozygote for haplotype IV. In addition, a patient who is doubly heterozygous for haplotypes I and VI is also homozygous for the IVS1 position 6 mutation.

The molecular lesions in the β-globin genes from four other patients were not detected by the above strategy. The β-globin genes of a patient homozygous for haplotype IX were cloned in Charon 4A and subcloned in pBR322. Supercoil DNA sequencing showed that the mutation was a G → C substitution at IVS1 position 5 (Fig 1). This substitution was subsequently confirmed by oligonucleotide hybridization to be present on both of this patient's chromosomes (Fig 2).

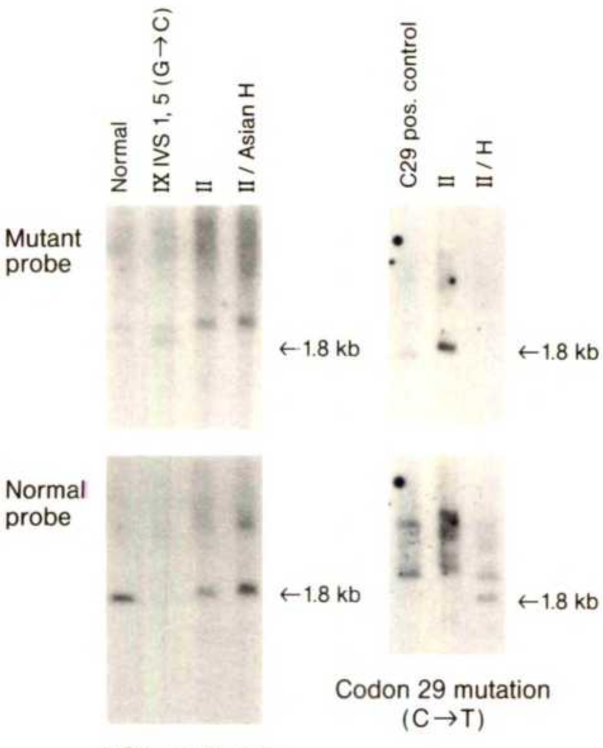
The second patient with an uncharacterized mutation was a double heterozygote for haplotypes II and Asian H. By oligonucleotide analysis, he carried the IVS1 position 110 lesion on one chromosome. His β-globin genes were cloned in Charon 4A followed by subcloning in pBS, and DNA sequencing revealed the IVS1 position 110 mutation on haplotype II. The other chromosome with the Asian H haplotype carried the dinucleotide deletion in codon 8, which has previously been reported to be associated with Mediterranean haplotype IV.

Two other patients homozygous for haplotype II failed to show any of the above mutations. The β-globin genes from one were cloned in Charon 30 and subcloned in pUC18. DNA sequencing revealed a base change (C → T) at codon 29 (Fig 1). Two oligonucleotide probes complementary to the normal and mutated sequence at codon 29 were then synthesized and hybridized to genomic DNA from these two

Table 2. Distribution of β-Thalassemia Haplotypes in Lebanon

Haplotype	ϵ	$^G\gamma$	$^A\gamma$	$\psi\beta$		β	$3'\beta$	Chrom	Frequency
I	+	−	−	−	−	+	+	31	0.62
II	−	+	+	−	+	+	+	7	0.14
III	−	+	−	+	+	+	−	2	0.04
IV	−	+	−	+	+	−	+	2	0.04
VI	−	+	+	−	−	−	+	3	0.06
VII	+	−	−	−	−	−	+	2	0.04
IX	−	+	−	−	+	−	+	2	0.04
Asian H	−	+	−	−	+	−	+	1	0.02

The polymorphic restriction sites were: The Hinc II site 5′ to the ϵ gene; the Hind III sites in the second IVS of the $^G\gamma$ and $^A\gamma$ genes; the two Hinc II sites in and 3′ to the $\psi\beta$ gene; the Ava II site in the β-gene and the Bam HI site 3′ to the β gene. (+) or (−) denote respectively the presence or the absence of the polymorphic site.



Codon 29 mutation (C→T)

IVS 1 position 5 (G→C)

Fig 1. Oligonucleotide hybridization of DNA from a normal patient and four patients in whom the mutations were not identified. In the left panels, oligo's 6 (see Table I) were hybridized to DNA from a normal individual, two patients homozygous for haplotypes IX and II, and one patient doubly heterozygous for haplotypes II and Asian H. Note that DNA from IX hybridized with the mutant probe, thus confirming the IVS1,5 (G → C) mutation. In the right panel, oligo's 6 were used. The first lane contains genomic DNA from a patient homozygous for haplotype II in which cloning and sequencing identified the codon 29 mutation and serves as a positive control. The second patient homozygous for haplotype II also hybridized with this probe. The patient with haplotype II/H did not hybridize with the mutant probe. The 1.8-kb fragments in which these two mutations reside are identified by the arrows. Other bands are due to cross-hybridizations.

patients. Both proved to be homozygous for the same substitution at codon 29 (Fig 2).

Thus in this sample of 50 β-thalassemia chromosomes from Lebanon, eight different mutations were found to account for β-thalassemia. Haplotype I and the IVS 1 position 110 lesion are strongly linked in this population and accounted for 60% of the β-thalassemia mutations. The other seven mutations are distributed at a frequency range of 2% to 8%. The heterogeneous β-thalassemia mutations in Lebanon can thus be detected by the use of six oligonucleotide probes and two restriction enzymes (Table 3).

Prenatal diagnosis. We have performed a prenatal diagnosis for a Lebanese fetus at risk for β-thalassemia by oligonucleotide hybridization of amniotic DNA. Previous screening of the parents and an affected child revealed that the β-thalassemia alleles were on chromosomes haplotype I, and hybridization with oligonucleotide probes showed that the mutation was the IVS1 position 110 (G → A). The fetal DNA when tested with oligonucleotide probes for this mutation hybridized only to the normal probe. Thus the fetus did not carry this mutant β-globin allele.

DISCUSSION

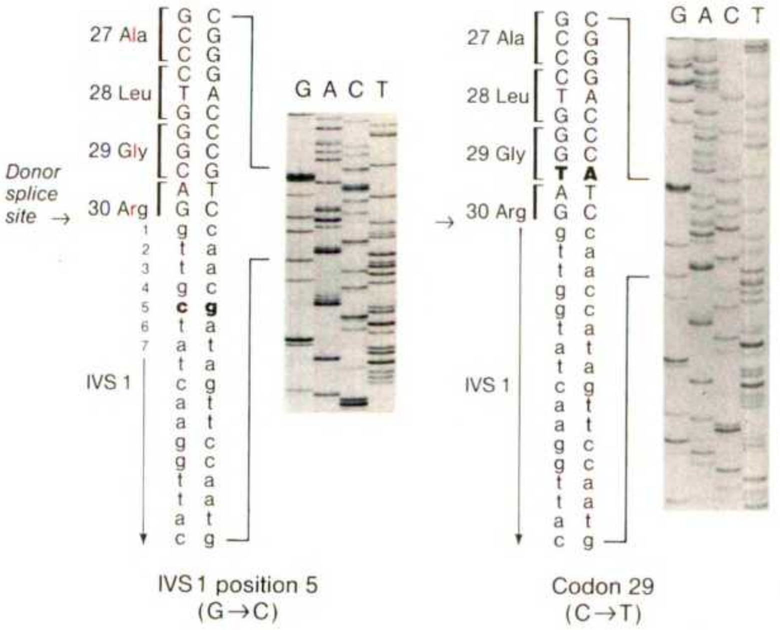
The clinical manifestation of β-thalassemia in Lebanon is severe, and homozygous patients have β/α globin synthetic ratios of 0 to 0.34.[1] In this study we have delineated the molecular defects in 25 transfusion-dependent β-thalassemia Lebanese patients by Southern blotting, synthetic oligonucleotide probing of genomic DNA, and molecular cloning followed by DNA sequencing. The Lebanese population carries eight different β-thalassemia mutations, six of which have been previously reported in the Mediterranean area. The IVS1 position 110, IVS1 position 6, IVS2 position 1, IVS2 position 745, β^{39} nonsense mutation, and frameshift mutation at codon 8 have all been shown to significantly alter the normal expression of the β-globin gene.[12-14] The linkage of β-thalassemia mutations in Lebanon to DNA haplotypes in the β-globin cluster was similar to that described[7] for haplotypes I, II, III, IV, VI, and VII. In addition we found four new linkages in this population: the mutation in IVS1 position 6 to haplotype I, the mutation at IVS1 position 5 (G → C) to haplotype IX, the dinucleotide deletion at codon 8 to the Asian H haplotype new to the Mediterranean, and the new mutation at codon 29 to haplotype II.

All of these mutations except the one at codon 29 have been previously described. The β^{39} mutation[13,14] has also been found in Sardinia,[15] Morocco, Algeria,[13] Greece, and Turkey.[16] The nucleotide substitution at the 5′ splice site of IVS2 has been described in an Italian and in some Iranian patients.[16] The dinucleotide deletion at codon 8 was originally detected in a Turkish patient.[7] The IVS1 position 110 mutation[17,18] is the most common cause of β-thalassemia in Mediterranean countries.[7]

The IVS1 position 6 mutation was originally found in a Portuguese patient[19] and subsequently in Greek Cypriots.[20] Typically the clinical course of patients with this mutation is mild in nature. However, the two Lebanese patients with this mutation have a more severe clinical disease as they both showed typical thalassemic facies, had β/α globin synthetic ratios of 0.34 and 0.20, and required regular blood transfusions when their hemoglobin levels fell below 6 to 8 g/dL. Although the reason for the more severe clinical course in the Lebanese patients is not known at this time, Lebanese parents at risk for this mutation should also receive genetic counseling for prenatal diagnosis.

The substitution (G → C) at IVS1 position 5 is interesting because it was previously found in the Chinese[21] and Asian Indian[22] populations. With its occurrence also in the Mediterranean region, it has now been found in three different populations. The fact that this mutation lies on a different haplotype in each population suggests that it arose independently in each and that this region of the β-globin gene is a hot spot for mutation.

The substitution at codon 29 was found in two patients, and while silent at the amino acid level, it creates a GT dinucleotide at this position 2 bp 5′ to the normal GT donor


27 Ala
28 Leu
29 Gly
30 Arg
Donor splice site →
1 2 3 4 5 6 7
IVS 1
GCCCTGGGCAGgttgctatcaaggttac
CGGGACCCGTCcaacgatagttccaatg
GACT
27 Ala
28 Leu
29 Gly
30 Arg
IVS 1
GCCCTGGGTAGgttggtatcaaggttac
CGGGACCCATCcaaccatagttccaatg
GACT

IVS1 position 5
(G→C)

Codon 29
(C→T)

Fig 2. DNA sequencing gel showing the mutations at IVS1 position 5 and codon 29. The horizontal arrows in both panels indicate the donor splice site, and the bold letters indicate the nucleotide substitutions. The coding strands are shown in the left columns of the sequences.

splice site of IVS1. The new GT dinucleotide may act as a donor splice site, thus causing a frameshift. Alternatively, as in the case of hemoglobins E,[23] Knossos,[24] and a β-thalassemia found in a black patient,[25] this mutation may activate cryptic splice sites present in this region of the β-globin gene. We are currently investigating the precise splice point of the codon 29 mutation by DNA-mediated gene expression transfer experiments. Nevertheless, since the phenotype of this mutant is β^+, some normal splicing of this mRNA must remain.

Until recently, prenatal diagnosis of β-thalassemia using DNA analysis was usually performed by establishing linkage of polymorphic restriction sites along the β-globin cluster.[26,27] Currently synthetic oligonucleotide probes provide a more direct method for detecting the mutation in prenatal diagnosis.[9,28] The delineation of the molecular lesion would allow a rational approach to prenatal diagnosis in the Lebanese population. While haplotype I and the IVS1 position 110 mutation are predominant, parents and any affected children should be screened for specific β-thalassemia lesions and DNA polymorphisms in the β-globin cluster prior to an attempted pregnancy. This approach has been successful in the case reported herein.

The heterogeneity of β-thalassemia mutations in Lebanon may be explained by the history of this country, which has long been a crossroads of several civilizations. The ancient Phoenician culture has been well documented as a migratory civilization from Lebanon to various regions of the Mediterranean Sea and Africa and could have thus served as a genetic vehicle across populations contributing to the widespread nature of these mutations. Lebanon has witnessed the passage of many different populations in the last 500 years, from the Crusades to the Ottoman empire, and for the last 200 years has seen the convergence of various Moslem sects from neighboring Arab countries. This admixture of ethnicities may well be reflected by the genetic diversity of β-thalassemia mutations resulting from population migration and gene flow.

Table 3. Linkage of Haplotypes to β-Thalassemia Mutations in the Lebanese Population

Haplotype	Type	No. of Chrom	Mutation		Frequency	Detection
I	β^+	30	IVS1 position 110	(G → A)	0.60	Oligo 1
	β^+	1	IVS1 position 6	(T → C)	0.02	Oligo 2
II	β^0	2	Codon 39	(C → T)	0.04	Oligo 3
	β^+	4	Codon 29	(C → T)	0.08	Oligo 5*
	β^+	1	IVS1 position 110	(G → A)	0.02	Oligo 1
III	β^0	2	IVS2 position 1	(G → T)	0.04	Hph1
IV	β^0	2	Codon 8	(−2, AA)	0.04	Oligo 4
VI	β^+	3	IVS1 position 6	(T → C)	0.06	Oligo 2
VII	β^+	2	IVS2 position 745	(C → G)	0.04	Rsa I
IX	β^+	2	IVS1 position 5	(G → C)	0.04	Oligo 6*
Asian H	β^0	2	Codon 8	(−2, AA)	0.02	Oligo 4*

*These mutations were initially detected by cloning and sequencing.

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Localization of Chlorotetracycline Fluorescence in Human Polymorphonuclear Neutrophils

By Thomas D. Coates, Martine Torres, Jon Harman, and Vicki Williams

Chlorotetracycline (CTC) has been used in many cells as a probe for membranous calcium. In polymorphonuclear neutrophils (PMN), the changes in CTC fluorescence upon stimulation are considered to monitor an early event in the activation process. Using quantitative video-enhanced microscopy, we report that in resting cells about 80% of the CTC signal emanates from the perinuclear region of the cell, indicating that internal structures are labeled with CTC. Approximately 20% of the total CTC fluorescence is taken up in a compartment sensitive to mitochondrial inhibitors, which is not present in neutrophils depleted of nucleus and granules or cytoplasts. Upon stimulation PMN loaded with CTC exhibit a rapid, biphasic decrease in fluorescence that is dose dependent. The second phase of the response is not seen in neutrophil cytoplasts. These results suggest that internal stores of CTC are responsive upon stimulation and could account for the later decrease in CTC fluorescence, whereas the early phase of CTC changes represents the plasma membrane response.
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THE FLUORESCENT probe chlorotetracycline (CTC) has been employed in several cell systems to detect membrane-bound divalent cations.[1-4] Its fluorescence is enhanced in apolar regions and in the presence of calcium or magnesium.[3,5] The effects of calcium and magnesium can be distinguished on the basis of differences in excitation and emission spectra.[3,5-7] While CTC fluorescence correlates with the uptake of radiolabeled calcium in resting cells,[2,8] it may not accurately reflect calcium status in stimulated cells where divalent cations, membrane potential, and intracellular pH are changing simultaneously. Factors other than calcium may play a role in determining the ultimate magnitude of CTC fluorescence change[9]; however, it is clear that change in CTC fluorescence is one of the earliest events during the activation of polymorphonuclear neutrophils (PMN) by n-formyl-methionyl-leucyl-phenylalanine (FMLP).[4,10] Since CTC is lipophilic, it can partition in all membranes. To further understand the nature of the CTC fluorescence response, we studied the localization of CTC within intact PMN and PMN devoid of granules and nucleus (PMN cytoplasts [CP]). By using the CTC response in CP as a model for the plasma membrane-associated CTC change, we have dissected the biphasic CTC response curve in intact PMN into an internal and an external component.

MATERIALS AND METHODS

Reagents. Cytochalasin B (Sigma Chemical, St. Louis) was dissolved in dimethyl sulfoxide (DMSO) at 10 mg/mL and used at a final concentration of 5 μg/mL. Aliquots of stock solution of FMLP (Sigma Chemical, St Louis) were prepared at 10^{-4} mol/L in 1% DMSO and kept frozen. CTC (ICN, Cleveland) was prepared fresh daily at 50 μmol/L in the experimental buffer. A 40% stock solution of Ficoll (mol wt 70,000, Pharmacia Fine Chemicals, Uppsala, Sweden) was made in 0.025 mol/L Tris-HCL, pH 7.4 and was filtered into a sterile flask. The stock was further diluted to prepare the gradient in phosphate-buffered saline (PBS) with 5 mmol/L glucose, pH 7.4 (PBSG).[11,12]

Cell preparation. Heparinized venous blood was obtained from normal volunteers in accordance with the precepts established by the Helsinki Declaration and with the approval of the Human Experimentation Committee of Indiana University. PMN were isolated as previously described[11,13] by dextran sedimentation, followed by centrifugation through a Ficoll-Hypaque gradient. PMN were maintained in HEPES buffer until ready for use.

Cytoplasts preparation. PMN were resuspended in a 12.5% Ficoll solution and treated for five minutes at 37°C with 5 μg/mL cytochalasin B. The suspension was then layered on a discontinuous gradient (25%, 16%) whose components had been prewarmed at 37°C for three hours. Cytochalasin B was present throughout the gradient. The gradient was then centrifuged for 30 minutes at 81,000 g (middle of the tube) at a temperature close to 33°C. After centrifugation the enucleated PMN were collected at the interface between the 12.5% and 16% layers.[11,12] The purity of the cytoplasts preparation was monitored microscopically and enzymatically as described.[11] Their functional activity was tested by their ability to produce superoxide anion (O_2^-). Preparations that were not in the normal range for both enzyme content and O_2^- production were rejected.

Chlorotetracycline measurement. After preparation as described, cytoplasts (CP) or cytochalasin B-treated PMN were washed three times in normal saline and resuspended in HEPES buffer, pH 7.4 (10 mmol/L HEPES, 130 mmol/L NaCl, 10 mmol/L KCl, 0.5 mmol/L glucose, 0.75 mmol/L $CaCl_2$, and 0.5 mmol/L $MgCl_2$) at 5×10^7/mL. Two hundred microliters of 50 μmol/L CTC in 10 mmol/L HEPES buffer, pH 7.4, was added to 200 μL of either the CP or the PMN suspension, and the resulting mixture was allowed to incubate at room temperature in the dark for 20 minutes. An aliquot of this solution was transferred to a cuvette, and the basal fluorescence was monitored in a Perkin-Elmer model MPF-44B fluorescence spectrophotometer (Perkin-Elmer Corp, Instrument Division, Norwalk, CT) equipped with magnetic stirring and a thermostated cell holder. The final concentration of cells was 5×10^6/mL and that of CTC was 2.5 μmol/L. Excitation and

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emission wavelengths were 386 nm and 544 nm, with slits of 10 nm and 15 nm respectively. Results are expressed as percent decrease of CTC fluorescence with respect to baseline: $F_t \times 100/F_0$, where F_0 is the fluorescence baseline and F_t is the fluorescence at time t. To study the early and late phases of the CTC response, the percent decrease in CTC fluorescence was measured 20 seconds and seven minutes after addition of the stimulus.

Chlorotetracycline uptake. The rate of uptake of CTC into PMN or CP was determined by exposure to a fixed concentration of CTC for varying periods of time. CTC (25 μmol/L) was placed in 0.9 mL of HEPES buffer containing 5 mmol/L glucose into 1-mL microcentrifuge tubes at room temperature. At t = 0, 100 μL of a suspension of PMN or CP at 5×10^7 cells/mL were added. The cells were centrifuged immediately or after fixed periods of time and then washed by gentle addition of buffer without CTC. After centrifugation the supernatant was decanted, and the cells were resuspended in 1 mL of buffer devoid of CTC. The suspension was then sonicated for 30 seconds. This procedure gave similar results as extraction of CTC with ethanol (data not shown). The total fluorescence of the suspension was then measured. For comparison of PMN and CP, the respective curves were normalized based on the fluorescence at 20 minutes.

Mitochondrial inhibitors. PMN or CP were treated for five minutes at room temperature with 1 mmol/L potassium cyanide (KCN), 1 mmol/L sodium azide (NaN_3), or 10 μmol/L Rotenone prior to performing the uptake experiments. NaCl or DMSO was used as a control.

Video microscopy. Microscopic observation of PMN was carried out in a chamber designed to allow easy exchange of bathing medium while cells are under observation.[14] The chamber was maintained at 37°C by means of an Air Curtain stage warmer. PMN at 1×10^6 cells/mL were placed in the chamber and allowed to adhere to the cover slip. The chamber was placed on the fluorescence microscope and the fluid was exchanged for buffer containing 50 μmol/L CTC and allowed to incubate for 20 minutes. Experiments were performed on a Leitz Orthoplan microscope with Ploem illuminator set up in epi-fluorescence mode using the UV filter set (ex 340 to 380 nm, dichroic mirror 430, em $>$ 510 nm). Viability of PMN chosen for observation was determined by presence of spreading and evidence of spontaneous movement. While there was some autofluorescence seen at these wavelengths, the fluorescence of CTC-loaded PMN was considerably greater than background autofluorescence. To reduce cell damage and photobleaching of CTC by the UV light, images were obtained with exposure to light for brief periods, usually less than five seconds.

Image analysis system. Fluorescence was recorded with a Dage-MTI model 66 silicon intensified target video camera (Dage-MTI, Michigan City, IN) connected to a Panasonic NV-8030 time lapse video tape recorder (VTR). Images were digitized from video tape using a DS-65 digitizer board (The MicroWorks, DelMar, CA) and an Apple II+ microcomputer (Apple Computer, Cupertino, CA). The software used in this system is described elsewhere.[15] The image analysis techniques used here for determination of integrated optical densities are standard.[16]

Localization. PMN are viewed on a video monitor using the freeze-frame mode of the VTR (Fig 1). The cell of interest was outlined on the monitor with a moving cursor. The area outlined was then digitized and mapped at four to one ratio to a color monitor. This results in a four times enlarged image with each range of intensity represented by a different color (Fig 2). By adjusting the ranges of intensity, it was possible to arrive at an assignment such that a particular color corresponded closely to a particular structure in the original image. This technique was possible only because CTC, and therefore the major source of fluorescence, was confined to discrete structures in the PMN. The intensity information from the image was stored as a grey scale histogram (Fig 3) used for integration and quantitation of the fluorescence. The regions in the image are identified by the computer on the histogram. Thus by summing the area under a particular region in the histogram, it is possible to determine the approximate fluorescence of that region.

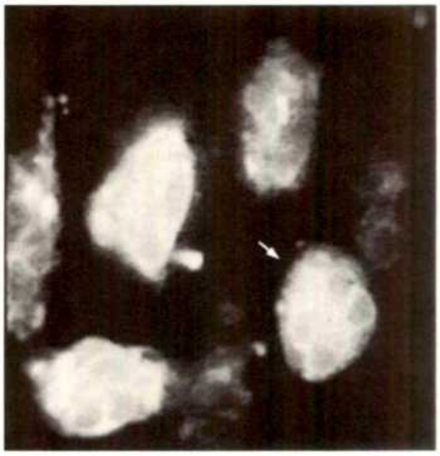

Fig 1. Video image of a group of PMN loaded with CTC. The image was photographed from the video monitor after recording on videotape. The cell indicated by an arrow corresponds to the cell digitized in Fig 2.

Data analysis. All data were analyzed using analysis of variance (ANOVA).

RESULTS

Video microscopy. When loaded with CTC, the fluorescence exhibited by PMN was not uniform. While present in all regions of the cell, the most intense fluorescence was seen in the perinuclear area between the lobes of the nucleus (Fig 1). One could observe discrete areas of bright fluorescence that moved and appeared to be attached to the nuclear membrane. These prominent fluorescent areas were adjacent to but not overlying the nucleus and thus represent CTC-stained membrane in the area between the lobes of the nucleus. Emanating from this same region were filamentous structures that fluoresced brightly with CTC (Fig 4). These areas were also brightly stained with the membrane potential probe rhodamine 123[17] (data not shown). When the cell formed a pseudopod, one or two of the filamentous structures could be seen moving out into the extended pseudopod and then retracting toward the center of the cell when the pseudopod retracted. These brightly fluorescent structures had clearly defined borders and were in the plane of focus of the nucleus. Some CTC fluorescence was present in a region that corresponds to the granules, as seen by transmitted light, and was located outside of the areas of focal fluorescence described above. When looking at CTC-loaded CP, which do not contain internal structures, a faint, uniform fluorescence was seen. Thus in PMN a significant part of the fluorescence emanates from membranous internal structures that were not present in CP.

Distribution of the fluorescence. When the frequency distribution of intensities from digitized PMN were examined, four regions could be identified (Fig 3). The brighter two regions corresponded to the areas of focal fluorescence,

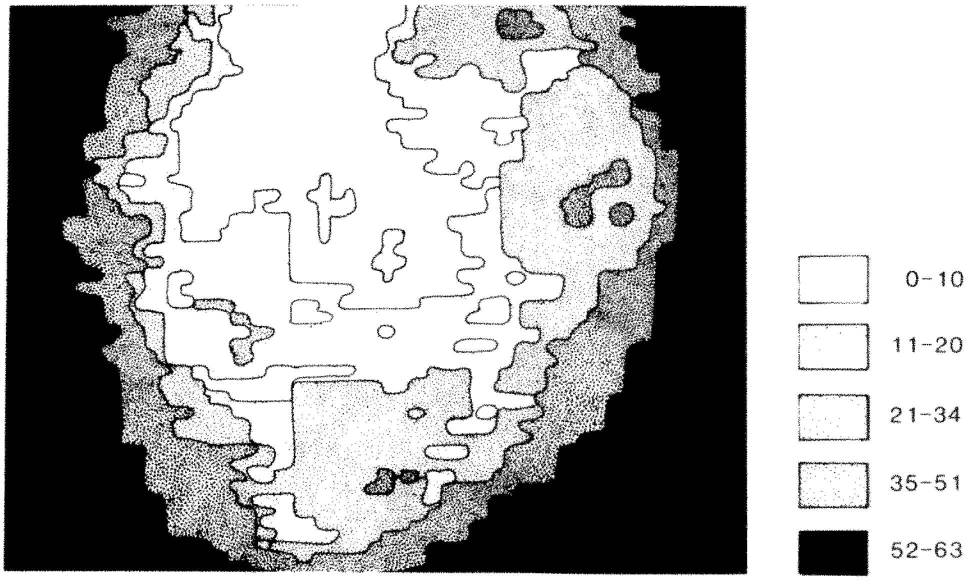

Fig 2. Intensity contour map of a cell from Fig 1. This map was produced from a pseudocolor representation. Note that 0 is the brightest and 63 is black. Region 0 to 10 is the perinuclear hot area, 11 to 20 the perinuclear membrane as well as dimmer areas of the perinuclear hot area, 21 to 34 a rough approximation of the area of the nucleus, and 35 to 51 the peripheral plasma membrane. Only part of the cell is seen because of the limitation of the display, but the digitization was done on the entire cell.

and the dimmer two regions to the area of the nucleus and of the plasma membrane. The percent of total fluorescence calculated from several cells by this method is shown in Table 1. Approximately 80% of the total cellular fluorescence came from the perinuclear area and nuclear membrane and occupied 60% of the image area. When corrected for the area of plasma membrane overlying the inner bright regions, plasma membrane fluorescence accounted for approximately 40% of the total cellular fluorescence. This is in agreement with the CTC baseline in suspensions of CP, which is 30% to 40% that of PMN at the same cell concentration.

Chlorotetracycline uptake. To assess possible differences in the compartmental distribution of CTC, the uptake of CTC was studied in similarly treated PMN and CP. Figure 5 shows the smooth uptake of CTC in CP achieving 50% of maximum by 60 seconds. In contrast, PMN demonstrated a biphasic pattern of uptake, reaching 50% maximum by approximately 10 seconds, followed by a slower rate from 60 to 1,200 seconds. The difference in pattern was highly significant ($P < 0.004$) when tested by analysis of variance. These data are consistent with the distribution of CTC in PMN in an additional compartment that is not present in CP.

Since CTC was localized in the same region as the mitochondrial probe Rhodamine 123, we investigated the effects of three inhibitors of mitochondrial function on the uptake of CTC. As demonstrated in Fig 6, KCN, Rotenone, and NaN_3 significantly inhibited the slow phase of CTC uptake ($P < 0.001$, 0.0001, and 0.007, respectively). When measured at 20 minutes, CTC fluorescence was inhibited by 24% with KCN, 21% with Rotenone, and 17% with NaN_3. Experiments were also carried out in CP that do not contain mitochondria.[18] Table 2 shows the CTC uptake after 20 minutes in CP treated with 1 mmol/L KCN or 1 mmol/L

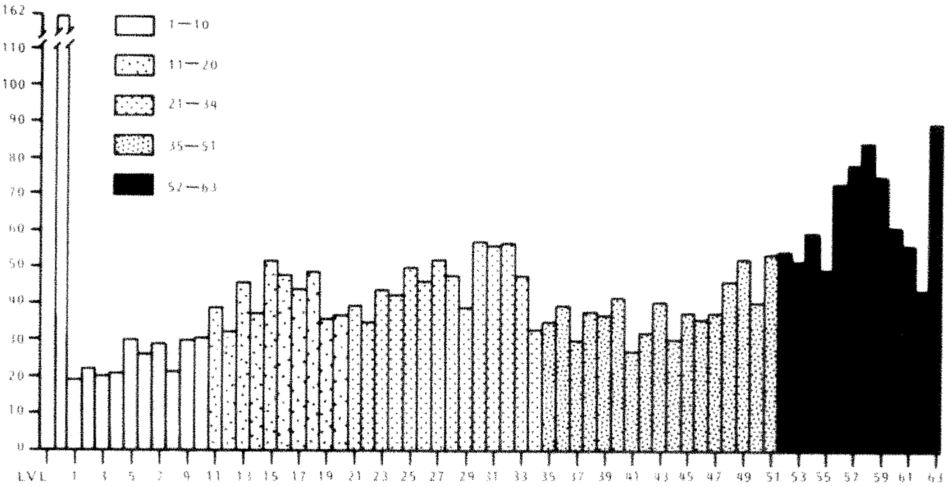

Fig 3. Video intensity histogram of the digitized cell in Fig 2. 0 is the brightest level and 63 the darkest level. The y axis represents the total number of points of a particular intensity in the image.

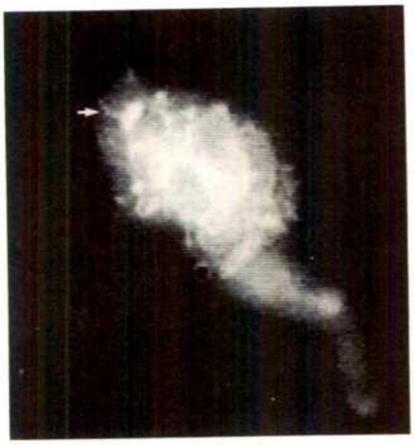

Fig 4. Image of a CTC-loaded neutrophil showing a bright filamentous structure (arrow).

NaN_3. There was no statistical difference in the amount of CTC uptake by CP ($P = 0.46$) in the presence or absence of these inhibitors. These data indicate that CTC uptake follows a distinctly different pattern in PMN and CP. Approximately 20% of the CTC is taken up into a compartment not present in CP that is sensitive to the mitochondrial inhibitors KCN, NaN_3, and Rotenone. While we cannot provide direct evidence that the CTC is in the endoplasmic reticulum and/or in the Golgi, 60% of the total fluorescence is in a nonmitochondrial inhibitor-sensitive, nonplasma membrane pool that is located in the perinuclear area of the cell.

Early and late responses in PMN and CP. The temporal pattern of fluorescence change in CP after stimulation with FMLP differs from that seen in intact PMN. Representative CTC fluorescence traces demonstrating these two patterns are shown in Fig 7. In PMN there was a rapid decrease in fluorescence during the first 20 seconds after stimulation, followed by a slower and more extensive loss of fluorescence. The second phase did not occur in PMN at doses less than or equal to 10^{-8} mol/L or at any dose in CP. Recovery was seen at low doses of stimulus and was not affected by the presence of CTC in the medium. Changes in fluorescence were measured 20 seconds and seven minutes from the time of addition of the stimulus to characterize the early and late responses when the cells were stimulated with increasing doses of FMLP. The early change in fluorescence was present in CP and did not significantly differ from that seen in PMN. There was no dose response in CP ($P < 0.19$), whereas a small but significant dose response was present in PMN ($P < 0.05$) (Fig 8A). When the late response was measured at seven minutes from stimulation, there was no further decrease in fluorescence in CP with no dose effect ($P < 0.03$) when a dramatic decrease was seen in PMN at seven minutes ($P < .0001$) (Fig 8B). This was not related to release of CTC in the external medium during degranulation, as no increase in CTC could be measured in the supernatant (data not shown). The secondary response was seen at doses of FMLP above 10^{-8} mol/L, similar to those usually associated with a secretory response.[19] These results imply that there is a second pool of CTC fluorescence, not

Table 1. Table of Regional Fluorescence

Region	% of Total Fluorescence	% of Total Area
I	28.66 ± 21.07	15.99 ± 16.95
II	52.64 ± 19.04	44.38 ± 18.96
III	16.20 ± 9.92	23.08 ± 11.34
IV	3.50 ± 2.19	16.50 ± 7.61
I + II	80.30 ± 10.72	60.37 ± 16.03
III + IV	19.70 ± 10.70	39.63 ± 16.03

Approximations of the relative fluorescence and area of four regions within CTC-loaded PMN. These represent averages from 10 cells on a single day.

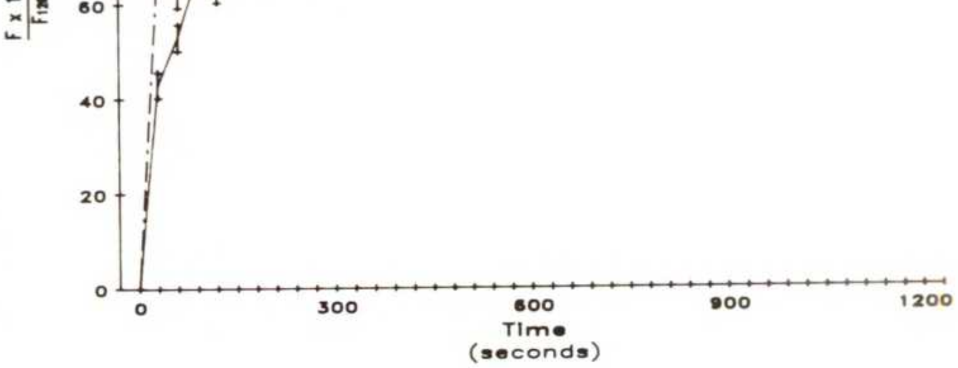

Fig 5. CTC uptake in PMN (----) and PMN-CP (——). The curves have been normalized and are presented as percent of maximal fluorescence. Total fluorescence of CP was 30% to 40% that of PMN at the same cell concentration.

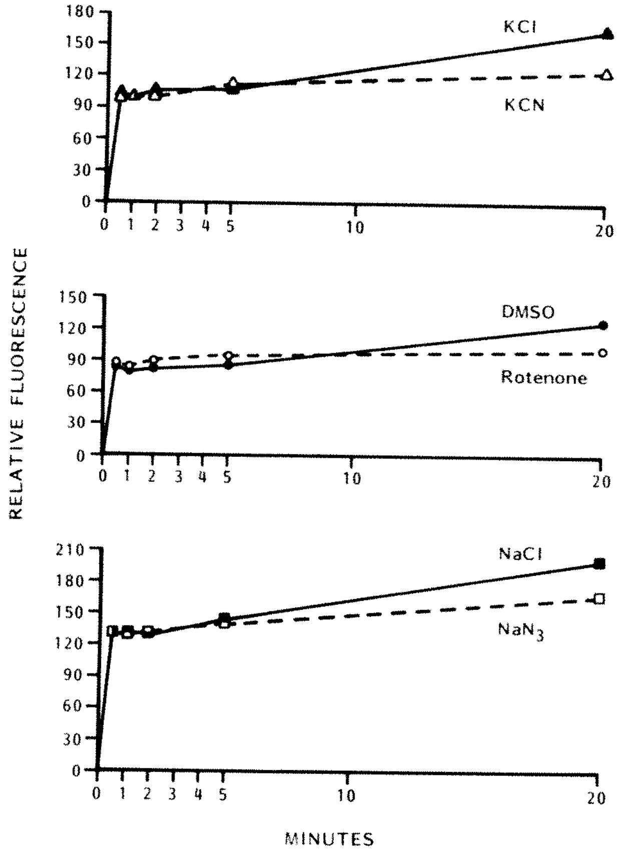

Fig 6. Uptake of CTC in intact PMN (———) and in the presence of mitochondrial inhibitors (------).

present in CP, that is released later after addition of stimulus at doses of FMLP greater than 10^{-8} mol/L.

DISCUSSION

Initial work using CTC fluorescence suggested that it represents a membrane-bound pool of calcium.[3,4,20,21] Change in fluorescence upon cell stimulation was interpreted as the release of trigger calcium[3,19]; however, the membranes labeled with CTC were not clearly defined. Direct visualization of CTC-labeled PMN presented here and by others[1,22,23] demonstrates that the fluorescence is located in the plasma membrane as well as in the perinuclear region of the cell, suggesting the existence of several membrane pools of CTC. Further evidence is given by the absence of internal focal fluorescence in PMN depleted of internal organelles and by the observation that in suspension PMN and CP have distinct patterns of uptake of CTC. Thus the compartmentalization of CTC observed microscopically exists in suspension and does not seem to be an artifact of adherence to glass slides.

The pattern of internal localization of CTC in PMN is consistent with the presence of calcium in mitochondria[24-26] and endoplasmic reticulum[8] as seen in other cell types. Mitochondria stained with CTC, as reported by others,[25,26] but only 20% of the CTC is present in this compartment. While mitochondria may play a role in the regulation of cytosolic calcium in PMN, the intracellular calcium concentration in PMN is more consistent with an endoplasmic reticulum-regulated calcium set point than with a mitochondria-regulated set point.[27] There is indirect evidence that the trigger calcium in PMN is located in an internal microsomal

Table 2. CTC Uptake in Cytoplasts

Treatment	% of Control	STD	p
Control	100		
NaN_3	98.5	13	NS
KCN	104.5	8	NS

Uptake of CTC in PMN cytoplasts after 20 minutes in the presence of 1 mmol/L KCN or 1 mmol/L NaN_3. The results are expressed as percent of control and are derived from the means of triplicates of five experiments.

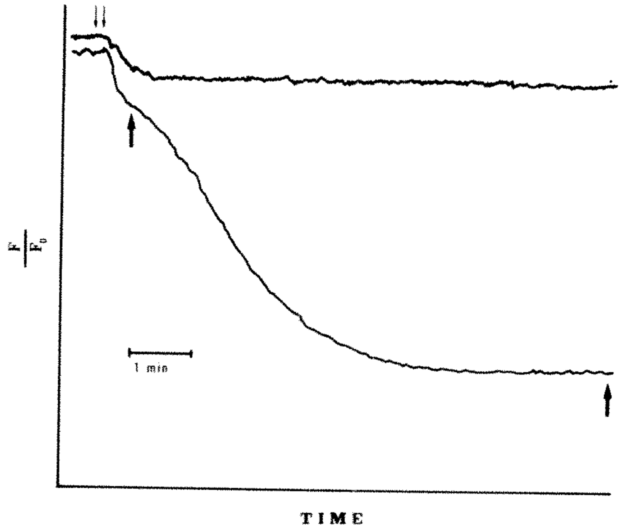

Fig 7. This tracing is representative of one experiment and shows the change in CTC fluorescence in PMN cytoplasts (top trace) and in PMN (bottom trace) when stimulated with 10^{-7} M FMLP (↓↓). Arrows indicate the times used for analysis in Fig 8 (↑).

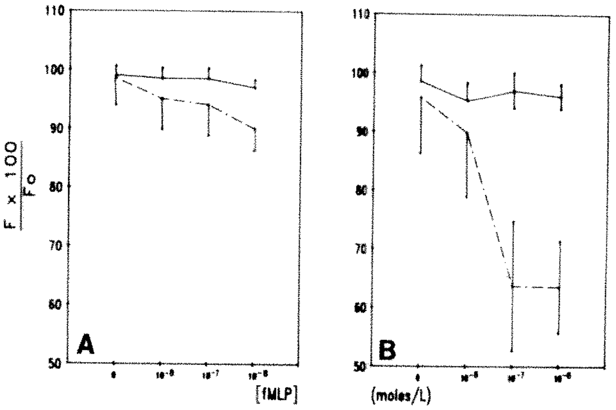

Fig 8. (A) demonstrates the CTC response 20 seconds from stimulation. There is no significant decrease in CP (———; P , 0.19), but the change in PMN is significant (-- --; $P < 0.05$). At seven minutes (B) there is still no dose effect in CP (———; $P = 0.3$); however, a significant effect is seen in PMN (-- --; $P < 0.0001$). Error bars represent the upper and lower 95% confidence intervals.

pool that is sensitive to inhibitors of the endoplasmic reticulum calcium pump.[27] The location of Golgi and endoplasmic reticulum in PMN corresponds spatially to the distribution of CTC seen in fluorescence microscopy. In other cell types the regions of perinuclear CTC fluorescence have been directly identified as Golgi and as endoplasmic reticulum.[23] Lysosomal granules represent another possible pool of intracellular calcium.[28] While some fluorescence comes from the granule membranes, the areas of maximum fluorescence are more centrally located. This is in agreement with another study using chicken PMN with large and more visible granulations.[22] These data support the hypothesis that a significant part of the CTC fluorescence in human PMN comes from internal pools.

Using CP as a model for neutrophil plasma membrane, the CTC response of intact PMN and of CP were investigated to determine if the internal pools of CTC were responsive upon stimulation with FMLP. Others have suggested that the major source of fluorescence loss is from the plasma membrane. Because of the technique used for microscopically studying the effect of stimulation, the earliest changes seen with soluble stimuli were observed at 60 seconds.[22] In suspension the early response is complete by this time, and the second phase of response is almost maximal. When the effects of stimulation are analyzed in suspension, there is little if any further response in CP at doses of FMLP greater than 10^{-8} mol/L, whereas in PMN there is a further phase of fluorescence loss. The biphasic CTC response seen in intact cells suggests that internal sources of CTC fluorescence, not available in CP, are responding to stimulation. The rate and dose dependency of the early response is similar in PMN and in CP and may be interpreted as change in the plasma membrane pool of CTC. The secondary response, which does not occur in CP, represents the CTC signal from internal sources.

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A Component of Factor VIII Preparations Which Can Be Separated From Factor VIII Activity Down Modulates Human Monocyte Functions

By Martha M. Eibl, Rafi Ahmad, Hermann M. Wolf, Yendra Linnau, Elisabeth Götz, and Josef W. Mannhalter

In this study we investigated different aspects of monocyte functions following interaction of monocytes (Mo) with therapeutic concentrations of factor VIII (F VIII) concentrate. A short (one-hour) treatment of normal Mo with F VIII concentrates led to a significant ($P < 0.001$) down modulation of Fc receptors expressed in the Mo plasma membrane. This down modulation was accompanied by a decrease of Mo effector functions that was expressed by a reduced capacity of F VIII-treated Mo to release O_2 radicals (40% of controls) and to kill bacteria (% killing: control Mo, 65%; F VIII-treated Mo, 24% to 51%). Further studies showed that the modulating activity was due to a contaminant present in F VIII concentrates (immune complexes or IgG aggregates). Fractionation using molecular sieving revealed that the modulatory activity was confined to a high-molecular range fraction (Mr > 1,270,000 daltons), while the fraction containing monomeric IgG had no effect. Further fractionation by affinity chromatography on protein A-Sepharose separated the coagulation activity (effluent) from the Mo function-modulating activity (eluate). We conclude that treatment with F VIII concentrates might contribute to an immunocompromised state in some hemophiliacs and facilitate opportunistic infections in these patients.

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SEROLOGIC surveys of hemophiliacs revealed a high prevalence of HTLV III/LAV antibodies in this patient population (70% and more of hemophiliacs in the United States and Europe are anti-HTLV III-positive).[1-3] It is generally understood that these patients develop antibodies in response to retrovirus transmitted by the respective blood products used for therapy.[4-8] However, the course of an infection is usually determined by both the infectious dose and the host's defense mechanisms. Thus an impairment of host defense mechanisms might constitute an additional risk factor and render the respective individual more susceptible to infections.

The macrophage/monocyte system is known to play a major role in the elimination of microbial agents. Recent experiments have shown that an impairment of macrophage functions (induced by silica, a substance known to be toxic to macrophages) increases the susceptibility of cats to feline leukemia virus, a retrovirus.[9] Furthermore, retrovirus particles have also been detected in monocytes of patients with acquired immunodeficiency syndrome (AIDS).[10] These data motivated us to evaluate monocyte functions following interaction of monocytes (Mo) with factor VIII (F VIII) concentrate.

In the present study we have investigated whether and to what extent treatment of Mo with F VIII concentrates affects Mo functions. We report that F VIII concentrates had the capacity to down modulate Mo functions, including expression of Fc receptors, generation of oxygen radicals, and intracellular killing of microorganisms. We were able to identify a contaminant in F VIII concentrate preparations as being responsible for the observed effects.

MATERIALS AND METHODS

Factor VIII concentrates. Lyophilized factor VIII (F VIII) concentrates (Kryobulin M, lot # 09M09083, Immuno AG, Vienna, Austria) were dissolved according to the instructions on the enclosed package insert (10 mL distilled water per 250 IU F VIII) and adjusted to the appropriate concentration in RPMI 1640 medium (Flow Laboratories, Irvine, England) supplemented with L-glutamine (2 mmol/L) and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin). Further definition of the F VIII concentrate used is given in Table 1.

Fractionation of F VIII concentrates. Fractionation of F VIII concentrates was first performed according to molecular size using gel chromatography. A Biogel A-1.5 M (Bio-Rad Laboratories, Richmond, CA) column (100 to 200 mesh, column size 2.6 × 95 cm) was loaded with 5 mL (89.2 IU) of F VIII and eluted at a flow rate of 27 mL/hour (elution buffer 0.024 mol/L sodium citrate, 0.12 mol/L sodium chloride, pH 7.2). This fractionation yielded five peaks with virtually all the F VIII activity confined to peak 1. The protein-containing fractions from peaks 1 to 5 were pooled, transferred into dialysis tubings (20 DM-X100 FT, Union Carbide, Chicago), and concentrated by embedding into polyethylene glycol 20,000 (Serva, Heidelberg, FRG) to an F VIII activity of 2 IU/mL in fraction 1. The volumes of fractions 2 to 5 were reduced to the same extent. The protein content of each fraction is shown in Table 2.

A part of fraction 1 (Mr > 1,270,000) was further subjected to affinity chromatography on protein A-Sepharose (protein A-Sepharose CL-4B, Pharmacia, Uppsala, Sweden; column size 1.6 × 4.5 cm, starting buffer 0.024 mol/L sodium citrate, 0.12 mol/L sodium chloride, pH 7.2; elution buffer 0.05 mol/L sodium citrate, 0.05 mol/L citric acid, pH 3.0; flow rate 28 mL/hour). The fractions eluting from the protein A-Sepharose column were immediately neutralized using 1 mol/L NaOH. The protein-containing fractions from both the protein A-Sepharose effluent and eluate were pooled and concentrated as described above.

Prior to use in cell culture experiments, all F VIII fractions were extensively dialyzed against Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}. For monocyte pretreatment studies, Ca^{2+} and Mg^{2+} ions were added immediately before the incubation step.

Poly IgG. Human serum IgG (Cohn fraction II, a kind gift from Immuno AG) was dissolved at a concentration of 10 mg/mL in Dulbecco's PBS containing Ca^{2+} and Mg^{2+} ions (Flow Laboratories) and polymerized by heat aggregation at 63°C for 20 minutes.[12] To remove large aggregates the preparation was then centrifuged at 600 × g for ten minutes.

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Table 1. Characterization of the F VIII Concentrate Studied

Total protein*	1896.0
Immunoglobulin*	
IgG	177.3
IgA	32.4
IgM	15.2
Specific activity (IU F VIII/mg protein)	0.53
F VIII R:Ag†	51.75
F VIII C†	23.8

Total protein content, immunoglobulin concentrations, and F VIII R:Ag were determined using standard procedures; F VIII C was assessed by a two-stage coagulation assay.[11]

*mg/1000 IU F VIII.

†IU/ml.

Isolation of mononuclear cells and preparation of monocyte monolayers. Mononuclear cells (MNC) were isolated from the peripheral blood of healthy human volunteers by buoyant density gradient centrifugation.[13] Anticoagulated blood (40 IU preservative-free heparin [Immuno AG, Vienna, Austria] per mL) was layered on Lymphoprep (Nyegaard & Co, Oslo, Norway) and centrifuged at 400 × g for 35 minutes. Then the mononuclear cells from the interphase were aspirated, washed three times in 0.15 mol/L NaCl, and suspended in RPMI 1640 supplemented with 15% pooled, heat-inactivated (30 minutes, 56°C) human AB serum, penicillin (100 IU/mL), streptomycin (100 μg/mL), and L-glutamine (2 mmol/L) (complete medium).

Monocytes (Mo) were prepared by adherence to plastic surfaces. MNC were suspended in complete medium at a concentration of 1×10^7 cells/mL. Two-milliliter portions thereof were pipetted into plastic tissue culture plates (Macro Tray 635 TC, Greiner & Söhne, Kremsmünster, Austria) and incubated at 37°C in a CO_2 incubator (5% CO_2 in humidified air) for 3 hours. At the end of the incubation period, nonadherent cells were removed, and the Mo monolayers were washed three times with 0.15 mol/L NaCl. Then complete medium was added, and the Mo monolayers were kept overnight at 37°C in a humidified CO_2 atmosphere. Adherent cells prepared in this way contained more than 90% Mo, as judged by nonspecific esterase staining.[14]

Pretreatment of Mo monolayers. Mo pretreatment was performed by incubating Mo monolayers with F VIII, F VIII fractions, or polymeric IgG for one hour at 37°C immediately after adherence. The F VIII concentrates were used at concentrations of 2 IU/mL, 5 IU/mL, and 10 IU/mL; protein concentrations of the respective F VIII fractions are shown in Table 2; polymeric IgG was applied at a protein concentration of 10 mg/mL. Mo pretreatment with buffer solutions (RPMI 1640 supplemented with L-glutamine and antibiotics and/or PBS containing Ca^{2+} and Mg^{2+} ions) was always included as a control. At the end of the 1-hour incubation step, the substances used for pretreatment were aspirated, and the Mo monolayers were washed with 0.15 mol/L NaCl and kept in complete medium at 37°C (CO_2 incubator) for 16 hours.

Determination of Fc receptors. Receptors for the Fc portion of IgG on the Mo membrane were determined by rosette formation with antibody-coated ox red blood cells.[15] Ox red blood cells were first sensitized with IgG by incubating equal volumes of ox red blood cells (2% suspension in PBS) and rabbit anti-ox red blood cell antibodies (IgG fraction, 0.167 mg/mL PBS, Nordic Immunology, Tilburg, The Netherlands) at 37°C for one hour.[16] Mo monolayers were washed twice with PBS containing 0.2% bovine serum albumin. Then the cells were gently scraped off with a rubber policeman and adjusted to 2 to 3×10^6 cells per mL. One hundred microliters of this cell suspension was mixed with 100 μL of a 0.5% suspension of IgG-sensitized red cells and centrifuged at 120 × g for ten minutes at 4°C. After 1 hour of incubation in an ice bath, the pellet was gently resuspended and the cells were examined by phase contrast microscopy. A minimum of 200 cells was counted, and a cell having three or more red cells attached was scored as a rosette. Results are expressed as percent rosette-forming cells (% RFC) or as attachment index (ie, the average number of red cells attached per Mo). The attachment index (AI) has been estimated by including all monocytes with none, one, or more than one erythrocyte attached and is calculated according to the following formula: AI = total number of red cells attached to 200 Mo divided by 200.

For competitive inhibition of rosette formation, 50 μL of Mo (6×10^6 Mo/mL) and 50 μL of IgG-sensitized red cells (1% suspension) were mixed with 100 μL of F VIII (2 IU/mL) or polymeric IgG (10 mg/mL) and treated as described above.

Determination of CR3. Expression of the complement receptor CR3 on monocytes was assessed by indirect immunofluorescence. Indirect immunofluorescence was carried out by first incubating (30 minutes, 4°C) 5×10^5 monocytes in 70 μL wash medium (Dulbecco's PBS containing 0.1% NaN_3 and 2% fetal calf serum) with saturating amounts of the monoclonal antibody (MoAb) Mol (94 ed, kindly provided by Dr R.F. Todd), which is directed against the iC3b receptor (CR3) on monocytes, granulocytes, and null cells.[17,18] After the first 30-minute incubation period, the cells were washed and again incubated for 30 minutes at 4°C in wash medium containing an excess of fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin (GAM-FITC, Coulter Immunology, Hialeah, FL). After a further wash step, flow cytometric analysis of monocyte cell-surface fluorescence was performed by using a FACS 440 cell sorter equipped with an argon ion laser operated at 488 nm and 270 mW and interfaced to a Consort 30 computer (Becton Dickinson, Mountain View, CA). Contaminating lymphocytes were excluded by preselecting a gate on the forward-angle light scatter *v* the

Table 2. The Modulatory Activity of F VIII Concentrates Is Confined to a High Mr Fraction

Mo Pretreated with	F VIII Activity	Protein Content (mg/dL)	% Rosette* Forming Cells	Attachment Index*
—	—		77 ± 3.5	3.16 ± 0.25
Dialysis buffer	—		74 ± 3.6	2.73 ± 0.20
F VIII, 2 IU/mL	+		38 ± 3.3	1.70 ± 0.17
F VIII fractions,†				
MR > 1,270,000	+	9.7	42 ± 4.1	1.91 ± 0.15
1,270,000-177,000	—	118.0	76 ± 3.0	2.86 ± 0.26
177,000-100,000	—	52.9	74 ± 4.1	2.74 ± 0.35
100,000-28,000	—	85.5	73 ± 3.8	2.70 ± 0.34
28,000-7,000	—	0.98	76 ± 2.5	2.76 ± 0.15

*$\bar{x}$ ± SD of three experiments.

†F VIII concentrate was fractionated by gel chromatography using Biogel A-1.5 M.

right-angle light scatter histogram; dead cells were excluded by their bright red fluorescence after ethidium bromide staining. A minimum of 10,000 cells was counted and the percentage of fluorescent-positive monocytes stained with the experimental antibody (Mol) minus the percentage of cells stained with a control reagent (mouse ascites containing antibodies directed against nonhuman tissue) was calculated.

Assays of monocyte effector functions (chemiluminescence and killing). Monocyte monolayers were washed twice with 0.15 mol/L NaCl. Then the adherent cells were gently scraped off with a rubber policeman and adjusted to 1 × 10^6 Mo/mL in Hanks' balanced salt solution (HBSS; for chemiluminescence) or HBSS supplemented with 1% gelatine (for killing assays).

Chemiluminescence of monocytes was determined according to the method of Trush et al.[19] In brief, 1 × 10^5 monocytes were pipetted into a plastic cuvette (Luma Cuvette, 3M, St Paul) and mixed with luminol (Lumanol-1000, Lumac, Medical Products Division/3M, St Paul) to give a final concentration of 63 μmol/L per cuvette. Then opsonized zymosan particles (Zymosan A, Sigma Chemical, St. Louis; opsonized by incubation in the presence of fresh serum [30 minutes, 37°C, shaking water bath]; 0.237 mg/cuvette) or an outdated intramuscular IgG preparation containing IgG aggregates (1 mg/cuvette) was added. The total volume per cuvette was 0.4 mL; all substances were diluted or suspended in chemiluminescence buffer (MEM-Dulbecco for chemiluminescence, Boehringer-Mannheim G.m.b.H., Mannheim, FRG). The resulting chemiluminescence was measured in a bioluminescence counter (Biolumat LB 9505, Berthold, Wildbad, FRG). The results of chemiluminescence intensity were plotted as counts per minute *v* time.

The capacity of monocytes to kill bacteria was assessed by a modification of a procedure originally described by Quie and coworkers.[20] Monocytes (1 × 10^5 in 100 μL) were pipetted into flat-bottomed microtiter plates (Microtest II, Falcon, Oxnard, CA) and mixed with *Escherichia coli* 089:H10 (monocytes to bacteria ratio = 2:1). Fresh AB serum (antibodies and complement)—essential for adequate opsonization—was added to give a total volume of 200 μL per microtiter well and a final serum concentration of 1:25. This suspension was incubated with continuous agitation for 90 minutes at 37°C. Incubation of bacteria in the presence of monocytes plus fresh AB serum absorbed with *E coli* to remove anti-*E coli* antibodies was used as a control. At the end of the incubation period the monocytes were lysed with distilled water, and the number of viable bacteria was determined by a plate-pouring technique. The number of bacterial colonies per plate was determined by using a colony counter (Biotran, New Brunswick Scientific Inc, Edison, NY).

Results are presented as the number of bacteria per plate ± SEM and as percentage of bacteria killed. This percentage was calculated according to the following procedure: the mean number of bacteria surviving in experimental wells was subtracted from the mean number of bacteria surviving in control wells. The difference was then divided by the mean number of bacteria surviving in control wells and multiplied by 100.[21] Control wells contained bacteria plus monocytes plus fresh serum depleted of anti-*E coli* antibodies; experimental wells contained bacteria plus monocytes plus fresh serum.

Statistics. Statistical evaluation of all results was performed using Student's *t* test or Student's *t* test for paired samples.

RESULTS

F VIII concentrate preparations have the capacity to down modulate Fc receptor expression on Mo from healthy subjects. The data from Fig 1 show that F VIII concentrates had the capacity to decrease the number of Fc receptors expressed on the surface membrane of the monocytes of healthy subjects (down modulation of the Fc receptor). Pretreatment of monocytes with F VIII concentrate preparations in concentrations within the therapeutic range led to a significant ($P < 0.001$) reduction in Fc receptor expression as assessed by both the decreased percentage of Mo forming rosettes with IgG-coated ox red blood cells (% RFC) and the reduction in the average number of IgG-coated ox red blood cells attached per monocyte (attachment index). The F VIII-mediated down modulation of Fc receptor expression was comparable to that obtained after pretreating Mo with polymeric (heat-aggregated) IgG, a substance known to down modulate Fc receptors[22] (controls [pretreatment with buffer]: % RFC 81 ± 1, attachment index 3.92 ± 0.16; pretreatment of Mo with F VIII [2 IU/mL]: % RFC 40 ± 1, attachment index 1.85 ± 0.09; pretreatment of Mo with polymeric IgG: % RFC 34 ± 3, attachment index 1.46 ± 0.16; results represent $\bar{x}$ ± SD of ten experiments). Fc receptor down modulation was further supported by the frequency distribution curve shown in Fig 2. The number of IgG-coated red cells attached per F VIII-

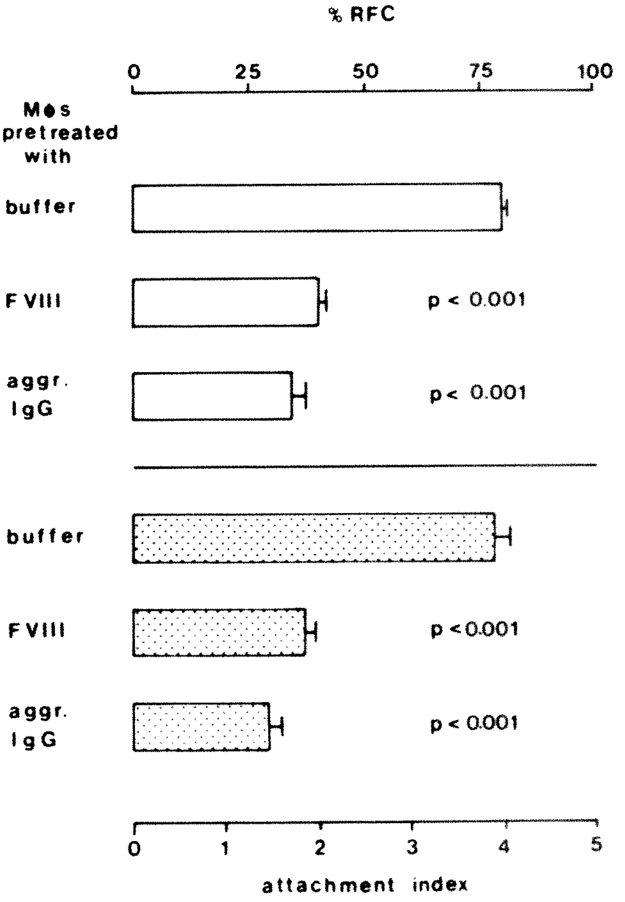

Fig 1. F VIII concentrates have the capacity to down modulate Fc receptor expression on normal monocytes. Normal Mo were incubated in the presence of buffer, F VIII (2 IU/mL), or polymeric IgG (aggr. 10 mg/mL) for one hour 16 hours prior to determination of Fc receptors. Each bar represents the percentage of Mo forming rosettes with antibody-coated red cells (% RFC) or the average number of red cells attached per Mo (attachment index).

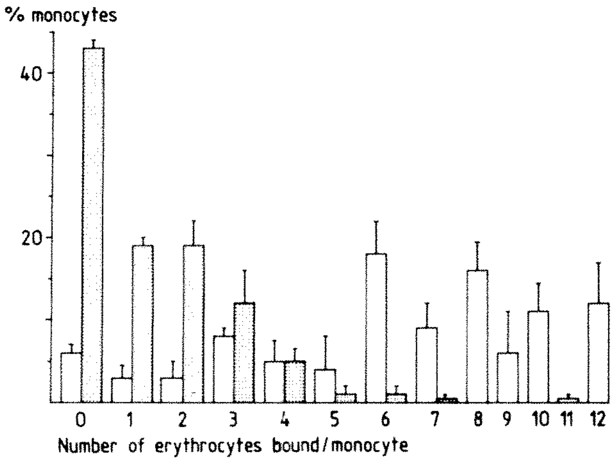

Fig 2. F VIII pretreatment of human monocytes leads to a reduction in the number of IgG-coated erythrocytes bound per monocyte. Human monocytes were pretreated for one hour with F VIII (2 IU/mL, dotted bars) or buffer (blank bars). After 16 hours FcR expression was assessed using IgG-coated ox red blood cells. The number of IgG-sensitized erythrocytes bound per monocyte was determined by phase contrast microscopy ($\bar{x} \pm$ SD of four experiments).

treated monocyte was substantially decreased as compared to the controls.

The decrease in Fc-receptor-positive Mo was achieved by both competitive inhibition of rosette formation (by adding F VIII to Mo together with IgG-coated red cells) and down modulation of the Fc receptor (by incubating Mo monolayers with F VIII for one hour 16 hours prior to rosette formation; Table 3). Our finding that sequential treatment of Mo with polymeric IgG and F VIII and vice versa did not further decrease the percentage of RFC or the attachment index (Table 4) strongly suggested that F VIII concentrates and polymeric IgG down modulated the same type of Fc receptor.

The reduction in the number of Fc-receptor-positive Mo could not be attributed to trivial cytotoxicity, since neither pretreatment with F VIII nor with polymeric IgG decreased the Mo's viability by more than 10%. Furthermore, the decreased expression of Fc receptors does not appear to be due to a blockade of the receptor by F VIII or by polymeric IgG. Studies using radioactively labeled immunoglobulins clearly demonstrated that after a 16-hour incubation period in the absence of IgG polymers, only minute amounts of polymeric IgG were found to be still monocyte-associated.[23] This 16-hour incubation period was always part of the Fc receptor-modulation protocol. Finally, a possible effect of buffer components present in lyophilized F VIII preparations was ruled out. Pretreatment of Mo with F VIII buffer (ie, buffer containing the same salt and amino acid concentrations as present in F VIII preparations after reconstitution of the lyophilized product with distilled water: 0.034 mol/L sodium citrate, 0.137 mol/L sodium chloride, 0.266 mol/L glycine) at a dilution corresponding to 2 IU of F VIII/mL did not alter the percentage of Fc-receptor-positive cells (control 84 ± 5.7, F VIII buffer 77 ± 6.6, F VIII [2 IU/mL] 51 ± 3.4; $\bar{x} \pm$ SD of four experiments).

Table 4. F VIII and Polymeric IgG Down Modulate the Same Type of Fc Receptor

Mo Pretreated with: First Pretreatment‡	Mo Pretreated with: Second Pretreatment‡	% Rosette* Forming Cells	Attachment Index†
Buffer	Buffer	86 ± 0.5	4.17
F VIII	Buffer	48 ± 1.1	2.57
F VIII	F VIII	53 ± 1.1	2.79
Poly-IgG	Buffer	44 ± 4.8	2.03
Poly-IgG	Poly-IgG	47 ± 5.3	n.d.§
Poly-IgG	F VIII	49 ± 3.1	2.05
F VIII	Poly-IgG	47 ± 4.5	2.21

*$\bar{x} \pm$ SD of six experiments.

†Average of two experiments.

‡Sequential one-hour pretreatments with the respective substances 16 hours prior to rosette formation.

§Not determined.

The F VIII concentrates and the respective buffer solutions used in this study were provided by Immuno AG, Vienna, Austria. Similar results with respect to down modulation of Fc receptors could also be obtained by using F VIII concentrates of varying purity (1.3 IU to 12 IU per mg protein) from several other manufacturers. Furthermore, no difference between heat-treated and non-heat-treated F VIII preparations could be observed (M.M. Eibl et al, manuscript submitted).

The Fc receptor-modulating component in F VIII concentrates can be separated from F VIII activity. The above mentioned data (F VIII-induced competitive inhibition of rosette formation and down modulation of Fc receptors

Table 3. F VIII Concentrate Leads to a Decrease in Fc-Receptor-Positive Mo by Both Competitive Inhibition of Rosette Formation and Down Modulation of the Receptor

	Competitive Inhibition of Rosette Formation*		Down Modulation of the Receptor†	
	% RFC‡	Attachment Index	% RFC‡	Attachment Index
Control	81 ± 1§	3.65 ± 0.33	81 ± 1	3.92 ± 0.16
F VIII (2 IU/mL)	39 ± 2.8	1.32 ± 0.20	40 ± 1	1.85 ± 0.09
Poly-IgG (1%)‖	30 ± 6.6	1.12 ± 0.20	34 ± 3	1.46 ± 0.16

*F VIII was mixed with monocytes and IgG-sensitized red cells immediately before rosette formation.

†Mo were incubated in the presence of F VIII for one hour 16 hours prior to rosette formation.

‡% rosette forming cells.

§Results are expressed as $\bar{x} \pm$ SD of ten individual experiments.

‖Poly-IgG served as a positive control in these experiments.

Table 5. The Fc-Receptor-Modulatory Activity of the F VIII High Mr Fraction Can Be Removed by Adsorption to Immobilized Protein A

Mo Pretreated With	F VIII Activity	Protein Content*	% Rosette† Forming Cells	Attachment Index†
Buffer (negative control)	—	—	79 ± 7.8	4.35 ± 1.03
F VIII (positive control)	2 IU/mL	1.27	44 ± 7.5	2.20 ± 0.44
High Mr fraction of F VIII	2.2 IU/mL	0.32	44 ± 9.7	2.28 ± 0.40
High Mr fraction not adsorbed to immobilized protein A (effluent)§	1.8 IU/mL	0.32	74 ± 3.3	3.84 ± 0.54
High Mr fraction adsorbed to immobilized protein A (eluate)‡§	—	0.08	43 ± 7.2	1.97 ± 0.47

All substances were dialyzed against Dulbecco's PBS prior to use.

*mg/mL.

†x̄ ± SD of four experiments.

‡Tested after elution with low pH.

§After passage through protein A-Sepharose, the volume of the effluent was reduced to give an F VIII activity of ~2 IU/mL. The volume of the eluate was reduced to the same extent.

comparable to the effects of polymeric IgG) already indicated the presence of a contaminating substance (immune complexes or IgG aggregates) in F VIII concentrate preparations. However, the possibility that the observed effects were due to steric hindrance of Fc receptors by F VIII binding to the monocytes could not be ruled out. Therefore F VIII concentrate preparations were subjected to fractionation. Molecular sieving using a Biogel A-1.5 M column yielded five protein peaks, and as the results from Table 2 show, the Fc receptor modulatory activity was confined exclusively to the high Mr fraction (Mr > 1,270,000 daltons). This fraction also included virtually all of the preparation's F VIII activity. None of the other fractions containing fibrinogen, monomeric IgG, or albumin had any effect on Fc receptor expression. The high Mr fraction of F VIII concentrate was then further fractionated by affinity chromatography on protein A-Sepharose, a substance known to interact with the Fc portions of human IgG_1, IgG_2, and IgG_4.[24] The data presented in Table 5 clearly demonstrate that only the fraction that could be adsorbed to immobilized protein A had the capacity to down modulate Mo Fc receptors (see Table 5, line 5). This fraction was also found to be completely devoid of F VIII activity. The fraction not adsorbed to immobilized protein A contained all the F VIII activity and had no Fc receptor-modulatory properties. Rough assessment using the F VIII preparation's total protein and IgG content as well as the protein concentration of the fraction bound to and eluted from immobilized protein A (protein A-Sepharose) indicates that about 1.9% of the total IgG in the F VIII concentrate used in this study was present in an aggregated and/or complexed form. Further results to be published elsewhere, however, demonstrate that the down-modulating activity of a given F VIII preparation is independent of the total IgG it contains (M.M. Eibl et al, manuscript submitted). Control experiments using Sepharose not complexed with protein A also demonstrated that the Fc receptor modulatory activity was not removed by passage through Sepharose alone (data not shown).

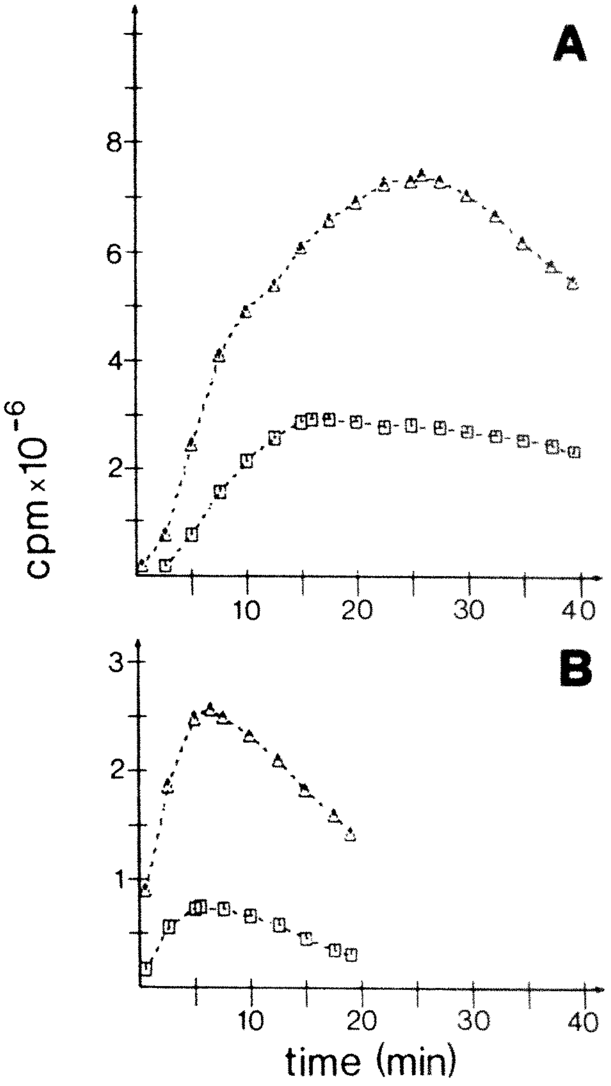

Fig 3. The capacity of human monocytes to secrete O_2 radicals is reduced by treatment with F VIII concentrate. Chemiluminescence induced by ingestion of zymosan (A) or IgG aggregates (B) was assessed 16 hours after pretreatment with PBS (△) or F VIII concentrate (□, 5 IU/mL). Chemiluminescence intensity measured in a bioluminescence counter is plotted as counts per minute (cpm) v time (minutes).

Effect of F VIII concentrates and F VIII concentrate fractions on monocyte effector functions. Since interactions with and ligation of Fc receptors are known to provide crucial triggering signals for intracellular microbicidal events,[25-27] it was of interest to determine whether the F

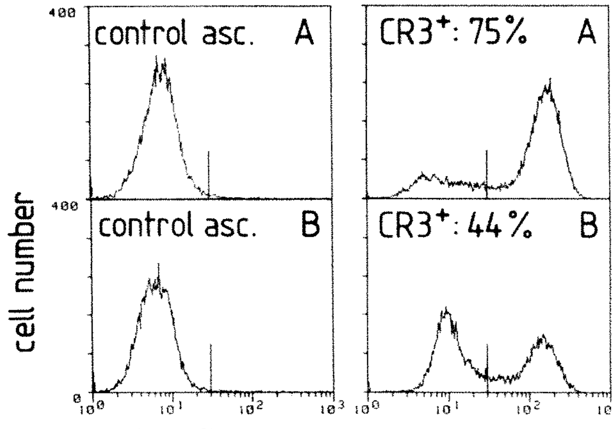

Fig 4. Interaction of F VIII concentrate with human monocytes leads to a decrease in the expression of CR3. iC3b receptor(CR3)-positive monocytes were assessed by indirect immunofluorescence and evaluated with a FACS 440 cell sorter. Sixteen hours after a one-hour pretreatment with buffer (A) or F VIII (2 IU/mL, B) monocytes were stained with the MoAb Mo1 (right panel) or with control ascites (left panel).

VIII-concentrate-mediated down modulation of Fc receptors would also modify monocyte effector functions. The data depicted in Fig 3 show that a one-hour pretreatment of Mo with 5 IU of F VIII concentrate 16 hours prior to the chemiluminescence assay led to a substantial reduction in the capacity of treated Mo to secrete O_2 radicals as compared to controls. The luminol-enhanced chemiluminescence was found to be reduced following stimulation of Mo by both opsonized zymosan and IgG aggregates.

The reduction of IgG aggregate-induced chemiluminescence can easily be explained by a decrease of Fc receptors in the Mo membrane. Chemiluminescence in response to zymosan is also decreased, but this substance stimulates the release of O_2 radicals by a pathway independent of Fc receptors, ie, following interaction with receptors for the complement cleavage product iC3b (CR3).[28] However, as can be seen in Fig 4, the expression of this receptor is also substantially diminished following preincubation of human monocytes with F VIII concentrate preparations. In that respect the Fc receptor-modulating component of F VIII concentrates behaves similarly to IgG polymers.[29]

In addition, pretreatment of Mo with F VIII concentrates also significantly down modulated these cells' capacity to kill microbes. *E coli* 089:H10, an *E coli* strain that requires opsonization by both antibodies and complement for proper elimination,[30] was used as a model system because this microorganism is easy to handle and is a potentially opportunistic pathogen. The data from Table 6 demonstrate that pretreatment of Mo with varying concentrations of F VIII concentrate preparations led to a dose-dependent reduction of the monocytes' bactericidal capacity (percent killing: controls [Mo pretreated with buffer] 64%, Mo pretreated with 2 IU/mL F VIII 50%, Mo pretreated with 5 IU/mL F VIII 34%, Mo pretreated with 10 IU/mL F VIII 22%). Similar results could be obtained if Mo were pretreated with fraction 1 (high Mr fraction [$Mr > 1,270,000$] eluting off the Biogel column after molecular sieving of F VIII concentrates). After pretreatment with fraction 1, the killing capacity of Mo was reduced to 36%, as compared to 64% obtained with control Mo (see Table 6, line 6).

DISCUSSION

Pretreatment of human monocytes with commercial F VIII concentrate preparations resulted in a down modulation of monocyte functions, as expressed by a reduction of Fc receptors and CR3 in the monocyte membrane, decreased release of oxygen metabolites following stimulation with opsonized zymosan and aggregated IgG, as well as impaired killing of bacteria.

Table 6. F VIII Pretreatment Reduces the Bactericidal Capacity of Human Monocytes

E coli Incubated in the Presence of	Mo Pretreated with*	No. of Bacteria Surviving†	% Killing‡
Complement, monocytes; no anti-*E coli* antibodies (control wells)	Buffer	731 ± 43	—
Antibodies, complement, monocytes (experimental wells)	Buffer	265 ± 10	64
Antibodies, complement, monocytes (experimental wells)	F VIII, 2 IU/mL	362 ± 43§	50
Antibodies, complement, monocytes (experimental wells)	F VIII, 5 IU/mL	485 ± 40‖	34
Antibodies, complement, monocytes (experimental wells)	F VIII, 10 IU/mL	571 ± 65‖	22
Antibodies, complement, monocytes (experimental wells)	Fraction 1 (2.2 IU F VIII/mL)	469 ± 21‖	36

*Mo were pretreated with buffer or F VIII for one hour 16 hours prior to determination of the monocytes' bactericidal capacity.

†Number of bacteria surviving after a 90-minute incubation period, $\bar{x} \pm$ SEM of eight experiments.

‡% killing was calculated according to the following formula:

$$\%\ \text{killing} = \frac{\bar{x}\ \text{number}\ E\ coli\ \text{in control wells} - \bar{x}\ \text{number}\ E\ coli\ \text{in experimental wells}}{\bar{x}\ \text{number}\ E\ coli\ \text{in control wells}} \times 100$$

Significant (§$P < 0.05$ and ‖$P < 0.001$) increase as compared to the number of bacteria surviving in the presence of monocytes (pretreated with buffer), antibodies, and complement.

The first observation was that F VIII preparations inhibited rosette formation when added to Mo together with IgG-coated red cells. Since IgG-coated red cells are known to interact with the receptor for the Fc portion of IgG,[15] these findings indicated a competition of two similar ligands for the same binding site. Furthermore, polymeric (heat-aggregated) IgG and F VIII concentrates behaved very similarly in competitively inhibiting rosette formation as well as in down modulation of the Fc receptor. This down modulation was achieved by a short (one-hour) treatment of monocyte monolayers with therapeutic concentrations of F VIII 16 hours prior to the respective assays and was comparable whether heat-treated or non-heat-treated F VIII preparations were applied. Sequential treatment of Mo with polymeric IgG and F VIII concentrates did not increase the effect. Fractionation of F VIII concentrate preparations then clearly showed that the modulating activity was confined to a high Mr fraction ($Mr > 1{,}270{,}000$), which showed no F VIII activity and could be purified by affinity chromatography on immobilized protein A.

The molecular nature of this (these) contaminant(s) can be assumed to be (an) immunoglobulin aggregate(s) and/or (an) IgG-containing immune complex(es). In this context it is important to note that, as has already been shown in other studies,[22,31] monomeric IgG (which can also be detected in commercial F VIII concentrates[32]) is completely inactive in modulating monocyte functions (see Table 2, line 7).

The presence of immune complexes or IgG polymers in F VIII concentrate preparations might have important clinical implications. In a previous study we were able to demonstrate that interaction of monocytes and polymeric (heat-aggregated) IgG led to a down modulation of the monocytes' capacity to release O_2 metabolites (in response to triggering by both aggregated IgG and zymosan) and to kill microbes.[29] In the present paper we report a comparable effect induced by a high Mr component present in commercial F VIII concentrates. Immune complexes or IgG aggregates have also been shown to interfere with immune defense mechanisms in vivo. Recently Virgin and Unanue[33] provided direct evidence for a correlation between the presence of circulating immune complexes and decreased resistance to infection. Using *Listeria monocytogenes* infection of mice as a model system, these authors demonstrated that in animals previously immunized with an unrelated antigen, secondary antigenic challenge led to a dramatically decreased resistance to the intracellular pathogen. The finding that the decrease in resistance could only be observed if antigenic challenge and infection were given together or not further apart than one day points toward an influence of immune complexes on the early phase of infection. The presence of high levels of circulating immune complexes has also been shown to be one of the major factors for the predisposition to viral and bacterial infections in patients with rheumatoid arthritis.[34] These in vivo data as well as the results from our in vitro studies thus make it conceivable that—together with the already increased level of circulating immune complexes frequently observed in hemophiliacs[35,36]—the continuous inoculation of immune complexes or IgG aggregates caused by F VIII concentrate treatment contributes to the hemophiliacs' increased susceptibility to opportunistic infections.

Recent clinical evidence indeed supports the possibility of an underlying immune deficiency in hemophiliacs.[37] Children with bleeding disorders who had accidentally been exposed to tuberculosis in a hospital ward showed an unusual susceptibility to that disease. These children, who had been regularly treated with F VIII concentrates, were almost equally as susceptible to tuberculosis as children receiving immunosuppressive chemotherapy (38% of the hemophiliacs developed tuberculosis as compared to 48% of the children on immunosuppressive chemotherapy). Furthermore, a defect with respect to antigen presentation by monocytes has been described in both anti-HTLV III-positive and anti-HTLV III-negative hemophiliacs,[38] an effect that could also be achieved by pretreatment of monocytes from healthy subjects with therapeutic concentrations of F VIII concentrates (manuscript in preparation).

The in vitro data reported in the present publication strongly suggest that treatment with F VIII concentrates might contribute to an impairment of host-defense mechanisms in hemophiliacs. The bleeding disorder that comprises the major clinical problem in hemophiliacs is mainly treated by application of F VIII concentrates. Despite the availability of heat-treated F VIII concentrate preparations, which more or less rule out a de novo infection with HTLV III,[39,40] the possibility of inducing an immunosuppressed state by this treatment should still be kept in mind. Live HTLV III has been found in the circulation of hemophiliacs,[41] and since the virus is difficult to detect, it might be present in many more of the patients but inhibited from multiplying by a still functioning immune system. This delicate balance could be impaired by F VIII preparations contaminated with immune complexes and/or IgG aggregates. Therefore hemophiliacs now treated with heat-inactivated F VIII concentrate preparations should also be closely surveyed for immunologic functions and the possible outbreak of opportunistic infections. In addition, it appears desirable that F VIII concentrates free of immunosuppressive high mol wt contaminants should become available.

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The Regulatory Role of Interleukin 2–Responsive T Lymphocytes on Human Marrow Granulopoiesis

By Zeev Estrov, Chaim Roifman, Gordon Mills, Tom Grunberger, Erwin W. Gelfand, and Melvin H. Freedman

The effect of recombinant interleukin 2 (IL2) on marrow CFU-C colony formation was evaluated to define the role for T lymphocytes in human marrow granulopoiesis. The colony-stimulating factor (CSA) used in our experiments was found to contain IL2. IL2 depletion from CSA resulted in a reduction in CFU-C colony proliferation. Addition of exogenous IL2 caused an increase in CFU-C colony numbers in a dose-dependent manner. This increase could be prevented by anti-Tac, a monoclonal antibody (MoAb) to the IL2 receptor. Moreover, anti-Tac in the absence of exogeneous IL2 resulted in an overall decrease in colony numbers. Depletion of either adherent cells or T lymphocytes abolished the effect of IL2 and anti-Tac on colony growth. In the presence of IL2, re-addition of T lymphocytes to the T-depleted marrow or adherent cells to adherent cell-depleted marrow resulted in a significant increase in CFU-C colony numbers, whereas no significant effect was found when IL2-depleted CSA was used. Although T lymphocytes were not themselves essential for CFU-C colony growth, our studies indicate that IL2 and IL2-responsive T cells can regulate in vitro granulopoiesis.

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CLONAL PROLIFERATION in vitro of committed granulocyte and monocyte-macrophage progenitor cells (CFU-C) is stimulated by glycoprotein molecules known collectively as colony stimulating activity (CSA).[1] Human monocytes and macrophages have been found to produce and release CSA[2-4] and when cultured with T cells in the presence of stimulatory antigen were found to produce much more CSA than either T lymphocytes or monocytes cultured alone.[5-7]

Although T cell lines[8-10] as well as mitogen-[2,11,12] or antigen-[12] stimulated T lymphocytes are able to synthesize CSA, T cell subpopulations have been reported to induce different effects on bone marrow (BM) CFU-C proliferation. Some investigators report stimulation of CFU-C growth, others have demonstrated inhibition, whereas still others have failed to identify any effect.[14-19]

Interleukin 2 (IL2), a secreted product of activated T cells[20] that is required for the proliferation of T cells, can enhance T lymphocyte production of a variety of colony-stimulating factors.[21] In the present study we used recombinant IL2 and a monoclonal antibody (MoAb) that blocks T cell-IL2 receptor sites[22] to demonstrate that IL2 responsive-T lymphocytes can interact with cells of the monocyte lineage to regulate in vitro granulopoiesis.

MATERIALS AND METHODS

Preparation of cells. Heparinized (10 U/mL) bone marrow (BM) was obtained after informed consent from hematologically normal adult volunteers who had been advised of the necessary procedures and attendant risks. These studies were approved by the Human Experimentation Committee at our institution. The BM cells were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (200 × g, 4°C) for 20 minutes to remove neutrophils and red cells.

Adherent cell removal. The mononuclear cells were depleted of adherent cells by incubation on plastic Petri dishes with 10% fetal calf serum (FCS) in α culture medium for 90 minutes at 37°C. In this technique,[231] free-floating cells that did not adhere to the plastic dishes were removed by gentle pipetting and repeated vigorous washing of the plates with medium. This procedure was repeated until all free-floating nonadherent cells were removed.

The remaining adherent cells were then removed from the dishes by gentle scraping with a rubber policeman, washed twice, counted, and kept at 4°C. In the reconstitution experiments, 20% to 100% of the removed adherent cells were added back to 1×10^5 adherent cell-depleted BM cells.

Both cell fractions, adherent and nonadherent, were characterized on cytocentrifuge smears by counting more than 500 cells after Wright's stain, nonspecific (alpha naphthyl butyrate) esterase stain,[24] and immunocytochemistry[25] with anti-MY 4 MoAb (Coulter Immunology, Hialeah, FL) to identify monocyte-promonocyte cells. Using these techniques the nonadherent cell fraction that had been removed from the plastic incubation plates as described contained <3% monocytes. The adherent cell fraction consisted of >95% monocytes.

Double T cell depletion. Post-Ficoll BM mononuclear cells were incubated with neuraminidase-sensitized sheep erythrocytes (SRBC) at 4°C for two hours, followed by gradient centrifugation.[26] This procedure was repeated twice. Cells at the interface contained less than 0.5% T cells as assessed by AET-sensitized SRBC rosette formation[26] and using anti-Leu-4 and anti-Leu-5 monoclonal anti-T cell antibodies (Becton Dickinson, Mountain View, CA). T lymphocytes were obtained by recovery of rosetted cells from the pellet following lysis with Tris-buffered ammonium chloride. In the reconstitution experiments these T lymphocytes were washed, counted, and 25% to 100% of the removed T cells were added back to 1×10^5 T cell-depleted BM cells.

Lymphocyte removal using CAMPATH-1. BM cells obtained following Ficoll-Paque gradient centrifugation were then washed once in phosphate-buffered saline (PBS) and treated with a monoclonal rat antihuman lymphocyte antibody (CAMPATH-1) and complement, as previously described.[27,28] No residual T lymphocytes following this treatment were found by E rosette formation or by indirect immunofluorescence with anti-Leu-1, Leu-4, Leu-5 (Becton Dickinson, Mountain View, CA), or anti-T3 (Coulter Immunology).

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Interleukin 2 (IL2). Recombinant IL2, derived from cDNA for the human IL2 gene expressed in *Escherichia coli*,[29] was provided by Biogen Research Corp, Cambridge, MA. The IL2 was reconstituted and, to confirm its activity, was tested in a sensitive, highly reproducible microassay based on tritiated thymidine uptake into the murine T lymphocyte line, 2.8.2, maintained in IL2-dependent long-term cultures.[30] This line does not respond to growth factors other than IL2. The material was also compared to IL2 obtained from the Gibbon cell line MLA 144, which showed similar effects. The stimulatory activity of the recombinant IL2 was blocked by anti-IL2 antibodies,[31] confirming the specificity of its effect. The concentration of biologically active IL2 was tested, defined and expressed in units using the murine IL2-dependent T cell microassay, as before.[30]

Anti-Tac antibody. Monoclonal anti-Tac antibody[22] with specificity for IL2 receptors was provided by W. Greene (National Institute of Health, Bethesda, MD). Its effect on an IL2-dependent T cell line was reversible after removal of the antibody, and could be overcome by high concentrations of IL2. Hybridoma supernatant tested as a control for anti-Tac was inactive in this system as well as in the CFU-C culture assay. Anti-Tac was added at the initiation of culture in dilutions of ascites fluid ranging from 1:333 to 1:1333. For control, anti-Leu-1 (Becton Dickinson, Sunnyvale, CA), a monoclonal pan-T antibody of identical subclass as anti-Tac (IgG_{2a}) was added to cultures in final concentrations of 0.025 to 1.0 µg/mL.

Preparation of CSA. Nucleated peripheral blood (PB) cells from a normal donor were immobilized in 10 mL of 0.5% agar (10^6 cells/mL) in 100-mm culture dishes. The cells were plated with and without 20 U/mL of IL2 and overlaid with 15 mL of 20% FCS in α-medium. The dishes were incubated for seven days at 37°C with 5% CO_2 in air in a humidified atmosphere. The cell-free supernatant was harvested, washed free of agar, tested on normal BM to determine its maximal activity, and stored in aliquots at −20°C.[32]

The biological activity of IL2 in CSA was tested in the murine IL2 dependent T cell microassay.[30] Standard CSA prepared from unstimulated PB cells (CSA-std) was found to contain 8 U/mL of IL2. CSA-std was depleted of IL2 activity using an anti-IL2 MoAb (provided by Dr K. A. Smith, Hanover, NH) bound to Affi-Gel (Bio-Rad Laboratories, Richmond, CA).[31] This IL2-depleted CSA (CSA-dep), when tested for IL2 activity, now contained 2 U/mL. In some of the experiments, CSA-dep was supplemented by adding 6 units to 33 units of IL2 (CSA-supp).

Highly purified human recombinant CSA (GM-CSF) that had no IL2 activity was provided by Steven C. Clark, Genetics Institute, Cambridge, MA. The preparation contained 220 µg/mL protein with a specific activity of 2×10^6 U/mg. A unit was defined as follows: a final concentration of 1 U/mL gave one half maximal colony formation.

CFU-C assay. The CFU-C assay was performed as described.[33] Briefly, nucleated BM cells (10^5) were cultured in 0.8% methylcellulose, 20% FCS, α-medium, and 20% CSA. The CSA concentration of 20% was used because it yielded maximum CFU-C growth-promoting activity. In one control study, recombinant GM-CSF 2 U/mL was substituted for 20% CSA. The culture mixture was placed in 35-mm Petri dishes in duplicate and maintained at 37°C, with 5% CO_2 in air, in a humidified atmosphere. Colonies were counted after 14 days using an inverted microscope. A CFU-C colony was defined as a cluster of 40 or more cells consisting of granulocytes, monocyte-macrophages, or both. Microscopically, granulocytic colonies could be easily distinguished from pure macrophage colonies because of the presence of smaller cells and tighter clustering. Individual colonies were plucked from the cultures with a micropipette and spread on glass slides, and the cellular composition was confirmed microscopically using Wright's stain and nonspecific (alpha naphthyl butyrate) esterase staining.[24]

Statistical analysis. The probability of significant differences between colony numbers was determined by Student's *t* test.

RESULTS

Comparative studies were performed to determine marrow CFU-C colony forming activity of the three CSA preparations. As a control, IL2 (35 U/mL) alone was added to cultures without CSA. The mean number of CFU-C colonies/culture produced in the presence of CSA-std, CSA-dep, and CSA-supp was 55, 32, and 140, respectively. IL2 alone yielded 36 colonies/culture. In control cultures prepared without CSA and IL2, the mean number of CFU-C colonies was 17. The differences in colony numbers between cultures with CSA-std and CSA-dep and between those with CSA-std and CSA-supp were significantly different ($P < 0.01$).

To further delineate the augmentative effect of IL2 on CFU-C growth, BM cells were cultured in four separate experiments with and without increasing concentrations of IL2 (Fig 1). The cultures were prepared with CSA-dep to confirm that the expected increments in colonies were IL2 dependent. The stimulatory effects of IL2 on CFU-C colony growth were evident in a dose-dependent manner ($P < 0.03$ comparing the highest concentration).

Similar dose-response studies were performed in four experiments with the anti-IL2 receptor antibody, anti-Tac (Fig 2). These cultures were prepared with CSA-std. The inhibitory properties of anti-Tac were observed to be dose-dependent ($P < 0.03$ comparing the highest concentration). In control cultures, anti-Leu-1, the monoclonal pan-T antibody of identical subclass as anti-Tac, had no effect on CFU-C colony growth.

To exclude the possibility of a nonspecific interaction between the crude preparations of CSA and IL2 or anti-Tac, IL2 and anti-Tac dose-response studies were repeated in cultures containing only recombinant GM-CSF 2 U/mL as the growth factor (Table 1). As shown, IL2 significantly increased ($P < 0.02$ with 20 U/mL) and anti-Tac decreased CFU-C numbers ($P < 0.04$ and <0.05 at dilutions of 1:300

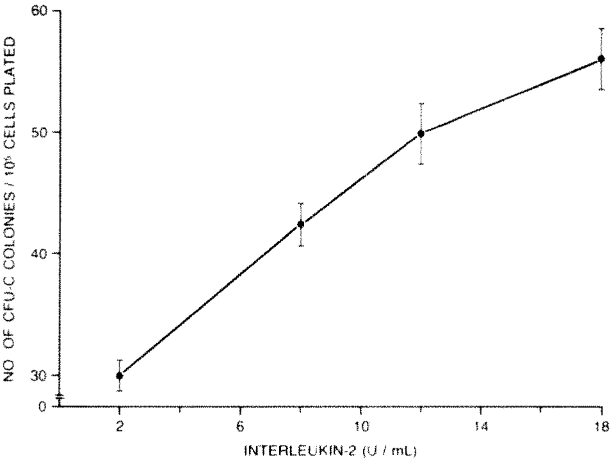

Fig 1. The effect of IL2 on CFU-C colony growth. Colony numbers from four experiments are expressed as mean ±SD. The CSA used in these studies (CSA-dep) contained 2 U/mL IL2. Increasing concentrations of IL2 were added to the CSA-dep.

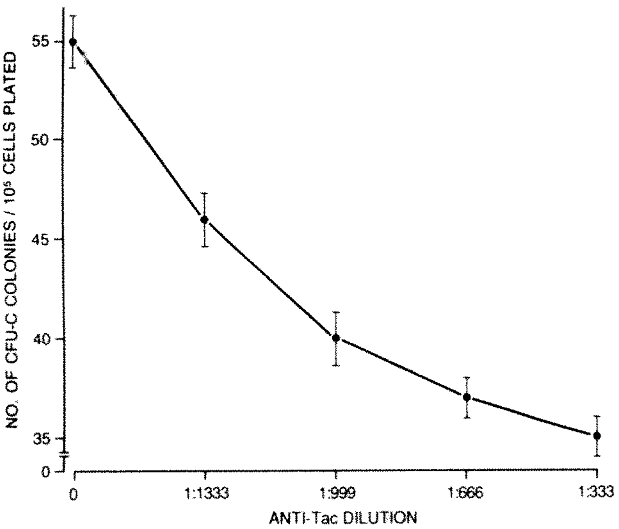

Fig 2. The effect of anti-Tac on CFU-C colony growth. Colony numbers from three experiments are expressed as mean ±SD. The CSA used in these studies (CSA-std) contained 8 U/mL IL2.

and 1:600, respectively) in a dose-dependent manner, identical to the previous studies.

To characterize which cell populations were involved in the modulatory effects exhibited by IL2 and the anti-Tac antibody, the effect of both agents was studied on BM samples that were depleted of T lymphocytes using CAMPATH-1. These studies were performed with CSA-std in the presence or absence of adherent cells (Fig 3). In the first column the stimulatory effect of IL2 on CFU-C colony numbers from post-Ficoll BM and the inhibitory effect of anti-Tac were both significant ($P < 0.01$ and $P < 0.025$, respectively). The removal from BM of either T lymphocytes (column 2) or the adherent cell fraction (column 3), or both (column 4), abolished the stimulatory property of IL2 and the inhibitory effect of anti-Tac.

To clarify the role of T cells in mediating the IL2 growth-promoting property on CFU-C colony formation, BM was depleted of T cells by rosetting and then reconstituted with varying T cell numbers (Fig 4). Using CSA-supp, no change in CFU-C colony numbers was observed until 50% of the removed T lymphocytes were added back to 1 × 10^5 T-depleted BM cells. Thereafter a T cell-dependent increase in CFU-C colonies was observed with a maximum increase over baseline of 100 colonies/culture when 100% of removed T cells were added back ($P < 0.001$). In control cultures with CSA-dep, no change in CFU-C colony numbers was seen on addition of T cells. Cultures of rosetted T lymphocytes plated alone with both preparations of CSA as control did not yield any CFU-C colonies.

The role of adherent cells on IL2-mediated CFU-C growth was examined in a similar manner. Adherent cells were

Table 1. The Effects of Recombinant Human GM-CSF, Interleukin 2, and Anti-Tac on Marrow CFU-C Colony Growth

Culture Conditions	CFU-C Colonies/Culture*
GM-CSF alone	50
GM-CSF plus IL 2:	
5 U/mL	50
10 U/mL	54
15 U/mL	69
20 U/mL	78
GM-CSF plus anti-Tac:	
1:1500	51
1:1000	51
1:600	38
1:300	35

All cultures contain GM-CSF 2 U/mL.

*Mean number of colonies from duplicate cultures is shown.

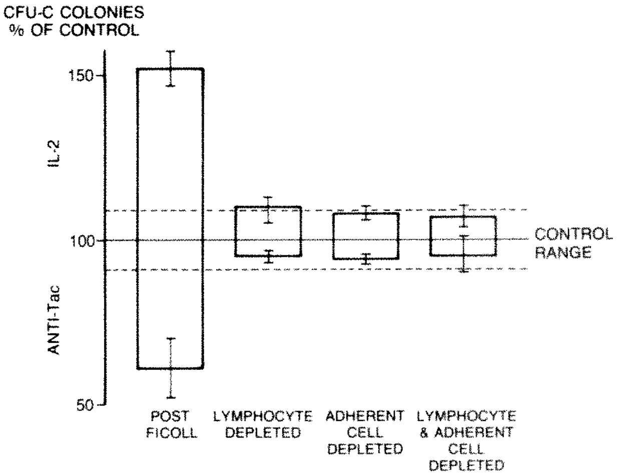

Fig 3. The effect of 15 U/mL IL2 and a dilution of 1:333 anti-Tac on four preparations of control BM. The data are from three separate experiments. CFU-C colonies are expressed as a percentage of the mean number of control colonies, which was defined as 100%. T lymphocytes were removed using CAMPATH-1. IL2 and anti-Tac were added to cultures using CSA-std containing IL2 8 U/mL.

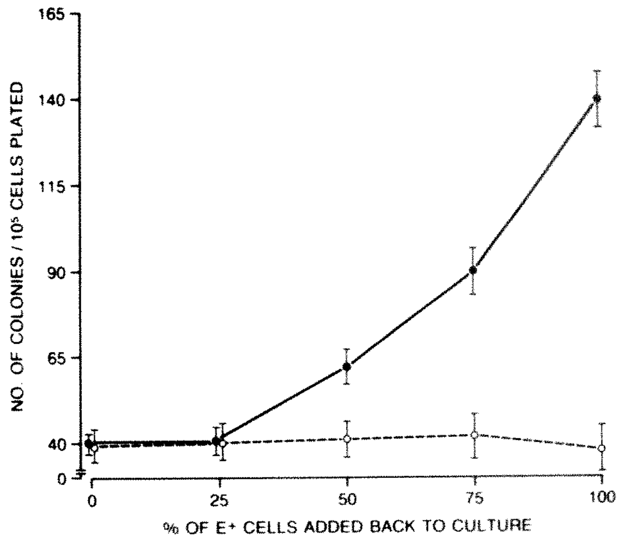

Fig 4. The effect of re-addition of rosetted T cell fraction to T-depleted BM in the presence of CSA-dep containing 2 U/mL IL2 (○—○) and CSA-supp containing 35 U/mL IL2 (●—●). Colony numbers are expressed as mean ±SD.

removed from the BM sample, and then added back to culture in varying numbers (Fig 5). Using CSA-supp, a significant increment in CFU-C colonies was found when 60% of the removed adherent cells were added to 1 × 10^5 adherent cell-depleted BM cells ($P < 0.01$) and plateaued ($P < 0.002$) when 80% to 100% of the removed adherent cells reconstituted the plated BM samples. No change in CFU-C colony numbers was seen in control cultures with CSA-dep on addition to adherent cells. Also, no CFU-C colonies developed when 2 × 10^5 adherent cells were cultured in the presence of CSA-std or CSA-supp.

To determine if IL2 could enhance CSA production from nucleated PB cells, the effect of CSA-std on CFU-C growth was compared to CSA prepared in the presence of IL2 20 U/mL (Fig 6). The BM was depleted of T cells and adherent cells prior to plating. At all four CSA concentrations tested, CFU-C colony numbers were higher in cultures containing the CSA prepared with IL2. At the highest CSA concentration tested, the difference in colony numbers between the two cultures was significant ($P < 0.01$).

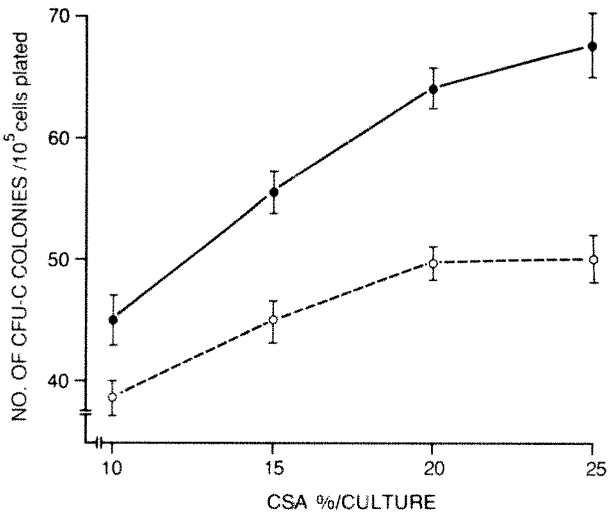

Fig 6. Comparison of four concentrations of CSA-std (O—O) and CSA prepared in the presence of IL2 20 U/mL (●—●) on CFU-C colony growth. The marrow sample was depleted of T cells and adherent cells prior to culture. Colony numbers are expressed as mean ±SD.

DISCUSSION

Previous studies have demonstrated the complicated cellular and humoral regulation of granulopoiesis.[2,8-19] A review of these publications, however, underscores many technologic problems, such as imperfect cell depletion methods, inadvertent enrichment of CFU-C when depleting other populations, and inability to quantitate or control interaction of the highly heterogenous subpopulations in normal marrow.

In the present study we examined the regulatory role on human marrow granulopoiesis by two BM subpopulations, IL2-responsive T lymphocytes and adherent cells. In an attempt to circumvent some of the technical problems that could flaw the study, we used recombinant IL2, a specific stimulator of activated T cells,[20,21] and the monoclonal anti-Tac antibody,[22] a blocker of IL2 receptor sites. Although other cell lineages are known to express receptor sites for IL2, only T lymphocytes are responsive to its action. Therefore IL2 and anti-Tac antibody in our studies were highly specific, and by stimulating or inhibiting an IL2-responsive T cell population, we were able to study the effect of these cells on CFU-C colony growth. The depletion of T lymphocytes by removal of E rosetting cells or by treatment with CAMPATH-1 abolished the effect of these cells on in vitro granulopoiesis, indicating that mature T lymphocytes can modulate CFU-C proliferation.

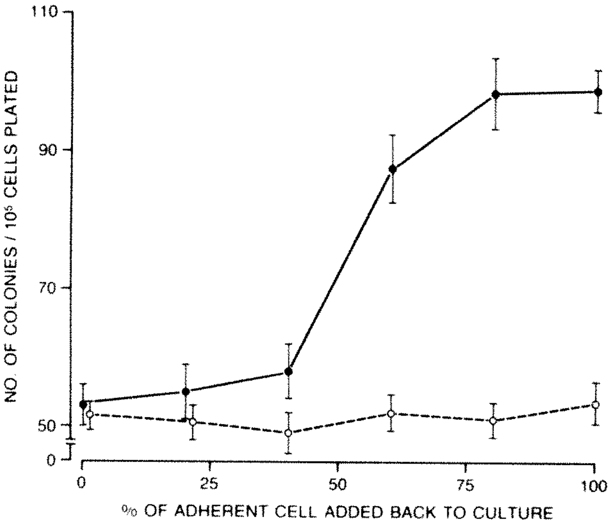

Fig 5. The effect of readdition of adherent cells to adherent cell-depleted BM in the presence of CSA-dep containing 2 U/mL IL2 (O—O), and CSA-supp containing 35 U/mL IL2 (●—●). Colony numbers are expressed as mean ±SD.

Our results showing IL2 enhancement and anti-Tac suppression of CFU-C colonies support previous reports suggesting that granulocyte-macrophage progenitor proliferation is controlled by T cells.[6,7,15,34,35] IL2-responsive T lymphocytes themselves have been reported to produce colony-stimulating factor,[21] but in our studies of fractionated BM, neither the IL2 nor the anti-Tac effects were observed when the adherent cells were removed from cultured BM cells. That is, IL2 and anti-Tac modulatory properties were expressed only in the presence of both BM adherent cells and T lymphocytes. Moreover, re-addition of T cells to the culture plates in the presence of 35 units IL2 and adherent cells increased CFU-C colony numbers in a dose-dependent manner. Similarly, re-addition of adherent cells to the cultures in the presence of IL2 and T cells increased CFU-C colony growth. These findings reflect the regulatory role of IL2 responsive T lymphocytes and the adherent cell fraction on CFU-C proliferation.

The requirement for BM adherent cells as well as T lymphocytes to mediate the modulatory properties of IL2 and anti-Tac on CFU-C growth contrasts with our findings for CFU-E and BFU-E proliferation.[36] Whereas IL2 resulted in an increase in marrow CFU-E and BFU-E colony numbers in a dose-dependent manner and anti-Tac had the opposite effect, identical to our CFU-C studies, depletion of marrow adherent cells did not alter the IL2 or anti-Tac effect on erythroid colony growth. Only the removal of marrow T lymphocytes blunted the effects of IL2 and anti-Tac on erythropoiesis. The data were consistent with the thesis that proliferating T cells released burst-promoting activity, (BPA) which, in turn, triggered erythroid progenitor proliferation.

In the CFU-C studies, the requirement for both cell populations suggests a different mechanism, probably one in which IL2-responsive T cells interact with adherent cells, which then elaborate CSA. This is strongly supported by our data on the CSA that was prepared from PB mononuclear cells in the presence of IL2 20 U/mL. That CSA preparation had significantly more activity on CFU-C growth than CSA-std when tested on T cell and adherent cell-depleted BM samples. Since IL2 did not stimulate CFU-C growth in the presence of either T cells or adherent cells but only in the presence of both, and since this CSA preparation was more active, we have to assume that a humoral factor that enhances CFU-C growth is released following the addition of IL2.

It had been previously reported that T lymphocytes and monocytes cultured together release more CSA than T cells or monocytes cultured alone.[5-7] Some investigators, however, failed to establish a clear role for T lymphocytes in either CSA production or regulation.[37] We have shown that CSA preparations contain IL2. Our standard CSA contained IL2 8 U/mL. Some investigators may not have appreciated this in the past. Clearly the IL2 content of CSA has important implications for marrow T cell replication and CFU-C growth. This was underscored by our study using highly purified recombinant GM-CSF that lacked IL2. The concentration of GM-CSF yielded maximum CFU-C colony growth, yet added IL2 augmented colony numbers even further. Using our CSA-std, the activity on CFU-C growth was reduced when IL2 was depleted and was enhanced by adding back IL2. These findings suggested an important regulatory role of IL2 in in vitro granulopoiesis.

Since in previous studies CSA was prepared in a similar manner, it is reasonable to assume that some of the conflicting results in the literature are due to dissimilar IL2 concentrations in different CSA batches. Motoyoshi et al[38] have suggested that some CSA species stimulate the BM adherent cells to produce endogenous CSA, which, in turn, causes CFU-C colony formation. This hypothesis may explain the ineffectiveness of IL2-responsive T cells on CFU-C growth in the absence of adherent cells.

T lymphocytes are not essential for CFU-C proliferation, since some CFU-C colony formation took place in the absence of T cells as well as in the presence of high concentrations of anti-Tac. Our findings, however, indicate that IL2, which is produced and secreted by T cells and has a central role in T cell function and immunity,[20,22,29,39] regulates in vitro granulopoiesis, probably by enhancing the interaction between T lymphocytes and adherent monocyte-macrophage cells.

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Prevention of Pulmonary Injury in Isolated Perfused Rat Lungs by Activated Human Neutrophils Preincubated With Anti-Mo1 Monoclonal Antibody

By G. Ismail, M.L. Morganroth, R.F. Todd III, and L.A. Boxer

Neutrophil activation results in neutrophil adherence and may subsequently cause lung injury through the generation of oxidants, release of granule proteases, and generation of a variety of mediator substances. We hypothesized that inhibition of neutrophil adherence and subsequent lung sequestration would attenuate the lung injury caused by activated neutrophils. Using isolated perfused rat lungs, we determined if anti-Mo1 monoclonal antibody (binds to the α subunit of a neutrophil glycoprotein [gp 155,94] that facilitates adherence) would attenuate lung neutrophil sequestration and lung injury caused by human neutrophils stimulated by phorbol myristate acetate (PMA). PMA-stimulated neutrophils but not PMA or neutrophils alone caused lung injury as assessed by accumulation of ^{125}I-bovine serum albumin into lung parenchyma and alveolar lavage fluid. Incubation of neutrophils with anti-Mo1 antibody prior to stimulation with PMA attenuated lung injury and neutrophil sequestration. Furthermore, a histological survey revealed that anti-Mo1 antibody inhibited neutrophils present in the lung from spreading following exposure to PMA. Anti-Mo1 antibody did not inhibit PMA-stimulated neutrophil release of granule constituents or toxic O_2 metabolites as evidenced by lysozyme and lactoferrin release or the reduction of ferricytochrome *c* in the lung perfusate. The inhibition of lung injury caused by the anti-Mo1 antibody was not likely due to a nonspecific effect of the antibody, since another murine monoclonal antibody of the same class (anti-Mo5) did not inhibit lung neutrophil sequestration or lung injury. Thus, in this experimental model, interference with the close approximation of the neutrophil to its target site inhibited the ability of the activated human neutrophil to cause injury.

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CONSIDERABLE EVIDENCE has accumulated over recent years suggesting that oxygen metabolites and proteases released by activated neutrophils may play an important role in the development of pulmonary inflammatory injury. Both oxidants and proteases have been detected in vivo in human disease and in models of pulmonary inflammation in experimental animals and isolated perfused lungs.[1-9] In experimental models of lung injury, acute lung damage can occur following a single dose of phorbol myristate acetate (PMA). This PMA-induced injury of rabbit lung vascular endothelial cells is dependent on the presence of neutrophils but the precise mechanism by which the neutrophil inflicts injury is not clearly understood.[10]

Following activation of neutrophils, not only do the cells release both proteases and oxidants, but they adhere more avidly to each other (neutrophil aggregation) and to artificial (plastic, glass) and physiological (endothelium, epithelium) surfaces.[11] Neutrophil adhesion which results in intimate contact between activated neutrophils and endothelial cells may be critical to the neutrophil's ability to inflict damage. These adhesion properties are necessary in order for activated cells to migrate on and through vascular endothelium in response to inflammatory stimuli. At the molecular level, progress has been made toward the identification of neutrophil surface glycoproteins that are involved in cell adhesion. Among these adhesion-promoting molecules is a heterodimeric glycoprotein, Mo1 (gp 155,95[12]) (equivalent to OKM-1[13] or Mac-1[14]) that also mediates the binding of C3bi-opsonized particles to the phagocyte membrane.[15] Monoclonal antibodies specific for the 155-kD alpha or 95-kD beta polypeptides of Mo1 have been shown to inhibit human neutrophil aggregation,[16,17] adhesion to plastic, glass, or endothelial cell surfaces,[18,19] and endothelial cell injury.[20]

With the availability of anti-Mo1 antibody the inhibition of neutrophil contact with its target can better be studied. We investigated in isolated salt-perfused rat lungs whether incubating human neutrophils with anti-Mo1 antibody prior to stimulation with PMA would inhibit lung neutrophil sequestration and subsequent lung injury. We also determined if anti-Mo1 antibody altered activated human neutrophil release of toxic oxygen metabolites or granule constituents. The isolated perfused rat lung was used in part since in this system we can concomitantly assess hemodynamic changes and the consequences of lung neutrophil activation (eg, sequestration, granule content release) over time. In this report, we demonstrate in isolated perfused rat lungs that PMA-activated human neutrophils induce acute lung injury which can be markedly attenuated by incubation of the neutrophils with anti-Mo1 antibody. The attenuation of the lung injury is not due to inhibition of neutrophil release of either superoxide or granule constituents. An identification of the mechanism by which neutrophils cause injury may lead to an understanding of the pathogenesis of some forms of the adult respiratory distress syndrome.

MATERIALS AND METHODS

Ex vivo lung preparation. An ex vivo model was used to assess lung injury and pulmonary vascular constriction following infusion of human neutrophils stimulated by PMA. Lungs were isolated from male Long-Evans rats (body weights 200 to 300g) which were anesthetized with pentobarbital (40 mg/kg intraperitoneally).

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Lungs were isolated and perfused at constant flow at 0.03 mL/g rat body weight/minute as previously described.[21] Mean pulmonary artery inflow pressure ($\overline{PA}$) was measured with a Statham (Oxnard, CA) transducer and recorded on a Grass (Quincy, MA) recorder. Perfusion pressure was proportional to pulmonary vascular resistance, since the flow for a given lung was held constant. Left arterial pressure (pulmonary outflow) was set by adjusting the height of the outflow reservoir and was kept at 0 cm H_2O. The lungs were ventilated via a tracheal cannula with an air/gas mixture containing 21% O_2 and 5% CO_2 at a rate of 60 strokes/minute using a Harvard animal respirator. The lungs and perfusate reservoir were kept at a temperature of 37° to 40°C.

Lung perfusion. Lungs were perfused from the outset of the experiment from a reservoir containing physiologic salt solution (70 mL) osmotically stabilized with Ficoll (4 g/100 mL). The first 30 mL of lung effluent was discarded to eliminate circulating rat blood elements from the vascular space of the lungs. This resulted in no neutrophils being detected in a 1:4 dilution of perfusate in 3% acetic acid. The lungs were then perfused for 30 minutes to reach a stable perfusion pressure and temperature prior to adding ferricytochrome *c* (50 μmol/L) to the recirculating perfusate. Ferricytochrome *c* was added to the perfusate so that its reduction could be used as an index of superoxide generation. Ten minutes after the addition of ferricytochrome *c* to the perfusate, human neutrophils were added to the lung perfusate. In some experiments catalase (1,500 units) was added to the lung perfusate. In other experiments, human neutrophils were incubated with either anti-Mo1 or anti-Mo5 antibody.

Isolation of neutrophils. Human neutrophils (98% neutrophils with >1% platelets/1,000 cells) were isolated according to the method of Curnutte and Babior.[22] This procedure resulted in 95% viable neutrophils by the trypan blue exclusion test. The cells were washed and resuspended in a final concentration in the lung perfusate of 3 × 10^6 cells/mL.

Incubation of neutrophils with monoclonal antibodies. The generation of murine monoclonal antibodies anti-Mo1 (IgG2a)[12,23] and anti-Mo5 (IgG2a)[24] has been previously described. IgG was purified from ascites fluid by *Staphylococcus* protein A–sepharose chromatography.[24] Saturating concentrations of anti-Mo1 and anti-Mo5, as determined by using indirect immunofluorescence flow cytometry,[12] were 1μg/10^6 human neutrophils. In experiments where cells were pretreated with antibodies prior to administration into the lungs, 1.2 × 10^8 cells in 1 mL phosphate-buffered saline (PBS) were incubated for 15 minutes at 37°C with antibody concentrations far exceeding saturation (600μg/mL). Following incubation, neutrophils were delivered into the lung perfusate at a final concentration of 3 × 10^6 cells/mL without prior washing (monoclonal antibody present in the perfusate). To confirm antibody excess sufficient to saturate Mo1 sites expressed by neutrophils after PMA activation, perfusates from selected experiments were assayed for the presence of residual soluble anti-Mo1 antibody by indirect immunofluorescence against neutrophil indicator cells.

Administration of PMA. PMA (50 ng/mL perfusate) was added to the perfusion reservoir 10 minutes after the addition of neutrophils to the reservoir. In control experiments the same amount of PMA was added to a reservoir which lacked human neutrophils. In addition, in a separate series of studies, lungs were perfused from a reservoir containing human neutrophils but to which no PMA was added. Mean pulmonary artery pressure was measured before and for 60 minutes following the addition of PMA. Pulmonary venous effluent was sampled before and 10, 20, and 30 minutes following the addition of PMA for determination of neutrophil counts, ferricytochrome *c* reduction, and granule constituent release as described below. The dose of PMA used was chosen because amounts of PMA in excess of 50 ng/mL resulted in fulminant lung injury and precluded completion of the experimental protocol.

Measurement of lung injury. ^{125}I-bovine serum albumin (^{125}I-BSA) was prepared by the standard chloramine T method.[25] Unbound iodine was removed by gel filtration using a G-75 column. ^{125}I-BSA was added to the perfusion reservoir 30 minutes after the addition of PMA. This was done in part so that pulmonary venous effluence could be sampled and assays performed on venous effluent that did not contain ^{125}I-BSA. Thirty minutes after the addition of the ^{125}I-BSA to the perfusate, 1 mL of pulmonary venous effluent was obtained, weighed, and counted for two minutes in a gamma scintillation counter. This allowed the determination of the number of ^{125}I-BSA counts in 1.0 g of circulating perfusate.

The reservoir supplying perfusate to the lung was then changed to contain only physiologic salt solution osmotically stabilized with Ficoll (4 g/100 mL). After the lung was perfused with 15 mL of this perfusate, 1.0 mL of pulmonary venous effluent was obtained and assessed for radioactivity in a gamma scintillation counter. This was done to confirm that minimal ^{125}I-BSA counts (<1% of the counts present in 1.0 gram of the circulating perfusate) remained within the intravascular space of the lung. The lungs and attached structures were then weighed prior to performing whole lung lavage, which was done by instilling 3.0 mL of normal saline into the tracheal cannula. This was instilled and withdrawn three times and 1 to 1.5 mL of total fluid was recovered. One mL of this lavage fluid was then counted for two minutes in a gamma scintillation counter to assess the amount of ^{125}I-BSA that accumulated in alveolar lavage fluid. The structures attached to the lung tissue were then dissected away from the lung and weighed. This weight was subtracted from the previously determined weight of the lung with attached structures to determine lung wet weight. The lung tissue was then counted for two minutes in a gamma scintillation counter to assess the amount of ^{125}I-BSA that had accumulated in lung parenchyma. ^{125}I-BSA counts present in alveolar lavage fluid, lung tissue, and pulmonary venous effluent were normalized by dividing the number of counts measured by the number of ^{125}I-BSA counts present in 1.0 g of circulating perfusate. The ratio of lung wet weight to the rat body weight was calculated as an index of lung water.

To assess neutrophil–pulmonary endothelial interactions in situ, a light and transmission electron microscopic survey of lung sections was performed comparing lungs perfused with neutrophils alone, neutrophils plus PMA, and neutrophils incubated with anti-Mo1 antibody plus PMA. Thirty minutes following the addition of the neutrophils to the perfusion reservoir the lung was fixed with 4% glutaraldehyde via tracheal instillation (at 15 cm H_2O). Sections (1 μ) were examined following staining of plastic-embedded tissue with toluidine blue.

Analysis of lung perfusate. The number of circulating neutrophils in the perfusate were counted on a hemocytometer. Perfusate samples that contained an undetectable number of cells at a 1:4 dilution in 3% acetic acid were considered devoid of circulating neutrophils. Ferricytochrome *c* reduction was used as an index of superoxide generation and was determined by optical density measurement of the perfusate at 551 nm. In separate experiments, it was demonstrated that the reduction of ferricytochrome *c* was abolished by the addition of superoxide dismutase (50 μmol/L) to the perfusate. Lysozyme activity (units/min/mL) and lactoferrin concentration (μg/mL) were measured as described previously.[26,27]

Assay for hydrogen peroxide generation. Hydrogen peroxide generation by resting and stimulated neutrophils was determined by the method of Root and Metcalf.[28] Neutrophils (2.5 × 10^6/mL) in Krebs-ringer-phosphate, pH 7.4, were incubated with 5 ng/mL PMA or 10^{-7} mol/L f-Met-Leu-Phe (FMLP) for 15 minutes. In some instances, 50 μmol/L ferricytochrome *c* was added to the buffer in order to evaluate the effect of this scavenger of superoxide on hydrogen peroxide formation.

Statistics. Values were expressed as mean ± SEM. Groups were

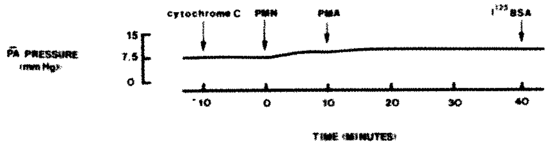

Fig 1. Typical tracing showing the experimental protocol and change in perfusion pressure following the addition of ferricytochrome c, human neutrophils (PMN), PMA, and ^{125}I-BSA (downward arrows) to a lung perfusate consisting of physiologic salt solution containing Ficoll.

compared using one-way analysis of variance and the Neuman-Keuls multiple comparison test.

RESULTS

Effect of anti-Mo1 antibody on lung neutrophil sequestration and lung injury. Addition of human neutrophils to rat lung perfusate containing physiologic salt solution in the absence of PMA caused $\overline{PA}$ pressure to increase 3 ± 0.4 mm Hg (n = 9, Fig 1). More than 95% of the neutrophils entering the lung were sequestered, since they were not recovered in the pulmonary venous effluent (Table 1, experiment A). Despite lung neutrophil sequestration, in the absence of PMA there was no increase in rat lung wet weight to body weight ratio or increase in accumulation of ^{125}I-BSA into lung parenchyma or alveolar lavage fluid (Fig 2, column two). Similarly, as seen in Fig 2, column one, PMA alone in the absence of neutrophils failed to cause increased rat lung weight to body weight ratio or increase in accumulation of ^{125}I-BSA into lung parenchyma or alveolar lavage fluid.

Neutrophils could not longer be detected in the lung effluent following the addition of PMA to lung perfusate containing neutrophils (Table 1, experiment A). Correspondingly, in these lungs the administration of PMA resulted in lung injury as assessed by increased rat lung weight to body weight ratio and increased accumulation of ^{125}I-BSA into lung parenchyma and alveolar lavage fluid (Fig 2, column three). This increased albumin accumulation in lung parenchyma and alveolar lavage fluid was not likely due to hydrostatic changes since the $\overline{PA}$ pressure measured during the period of albumin accumulation (12 ± 2 mm Hg) in these lungs (human neutrophils [PMN] and PMA) was not different (p = NS) from the $\overline{PA}$ pressure measured (10 ± 2 mm Hg) when only PMA was added to the lung perfusate. Generation of H_2O_2 was necessary for the lung injury to occur since addition of catalase (n = 5) to the lung perfusate prior to the addition of PMA inhibited the increased accumulation of ^{125}I-BSA in lung parenchyma (.36 ± .06, $P < .05$).

Incubation of neutrophils with anti-Mo1 antibody inhibited lung neutrophil sequestration both prior to and following the addition of PMA to the lung perfusate (Table 1, experiment B). Incubation of neutrophils with anti-Mo1 antibody had no effect on the $\overline{PA}$ pressure increase following addition of these neutrophils to the lung perfusate (3 ± .4 mm Hg, n = 6, P = NS). However, addition of PMA to lung perfusates containing neutrophils incubated with anti-Mo1 antibody resulted in less lung injury since the increase in lung wet weight to body weight ratio and accumulation of ^{125}I-BSA into lung parenchyma and alveolar lavage fluid were inhibited (Fig 2, column four). This inhibition of the lung parenchyma and alveolar lavage albumin accumulation was not likely due to hydrostatic changes, since the $\overline{PA}$ measured during the period of albumin accumulation (10 ± .6 mm Hg) in these lungs (PMN incubated with Mo1 antibody and PMA) was not different (P = NS) from that in lungs in which nontreated neutrophils were stimulated by PMA (12 ± 2 mm Hg). Lung albumin accumulation was also compared in a subset of lungs from each of these two groups. Lungs were paired such that the $\overline{PA}$ pressures during the period of albumin accumulation were identical (<.5 mm Hg difference). Comparing these subgroups (paired *t* test, n = 3) addition of PMA to lung perfusates containing neutrophils incubated with anti-Mo1 antibody, as opposed to nonincubated neutrophils, still resulted in less accumulation of ^{125}I-BSA into lung parenchyma (.43 ± .09 *v* 1.1 ± .1, $P < .05$) and alveolar lavage fluid (.03 ± .01 *v* .36 ± .3, $P < .05$). Treatment of neutrophils with anti-Mo1 antibody not only reduced the total number of cells sequestered in the lung, but anti-Mo1–treated neutrophils failed to spread on pulmonary endothelium after exposure to PMA as assessed microscopically (Fig 3, column C). In addition, the vascular endothelium appeared intact following exposure to PMA-stimulated neutrophils incubated with anti-Mo1 antibody. However, the anti-Mo1 antibody did not impair the development of vacuolization, a finding consistent with degranulation. In contrast, as seen in Fig 3, column B, PMA-activated neutrophils not exposed to anti-Mo1 antibody underwent spreading on endothelium, caused marked endothelial cell damage, and demonstrated vacuolization consistent with

Table 1. Circulating Neutrophils in Perfusate Following Infusion of Untreated or Antibody-Treated Human Neutrophils

	Before PMA Injection			After PMA Injection	
Experimental Conditions*	Neutrophils (× 10^5 Cells/mL)	Percentage Recovery†	P Value‡	Neutrophils (× 10^5 Cells/mL)	Percentage Recovery†
A. PMN alone	1.58 ± 0.63	4.9 ± 1.95		ND§	—
B. PMN-AbMo1	7.03 ± 1.07	23.3 ± 3.54	$P < 0.05$	0.36 ± 0.12	1.2 ± 0.39
C. PMN-AbMo5	2.50 ± 0.82	8.2 ± 2.68	P = NS	ND	—

*Neutrophils were added to the reservoir in a concentration of 3 × 10^6 cells/mL.

†Percentage recovery was calculated by dividing the number of neutrophils measured in 1 mL of pulmonary venous effluent by the number of neutrophils in the lung perfusate entering the lung.

‡P values represent comparisons between PMN alone and each of the other two conditions.

§ND indicates no neutrophils detected in a 1:4 dilution of perfusate in 3% acetic acid.

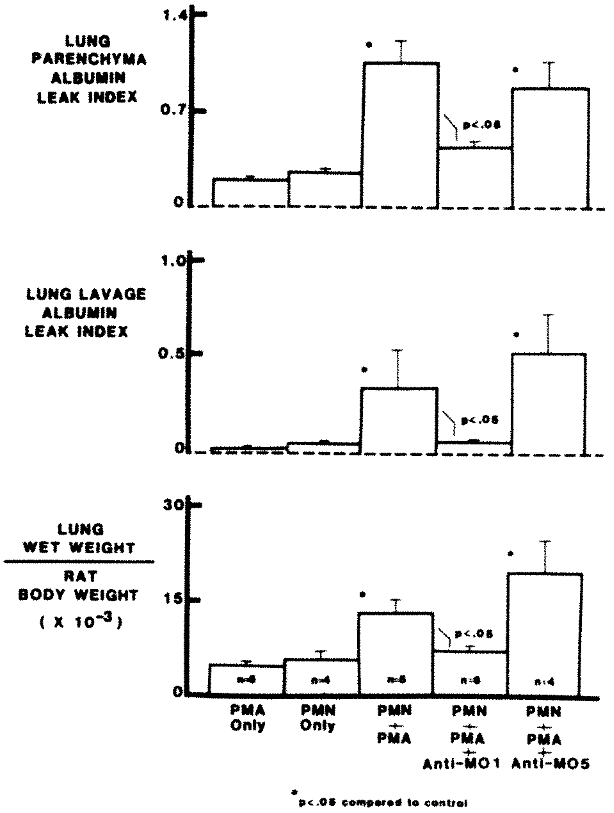

Fig 2. Effects on lung injury of PMA by itself (PMA only), unstimulated neutrophils (PMN only), and PMA-stimulated neutrophils with or without prior incubation with anti-Mo1 or anti-Mo5 monoclonal antibodies. Lung parenchyma and alveolar lavage albumin leak indices were calculated by dividing the ^{125}I-BSA counts in lung tissue or alveolar lavage fluid by the ^{125}I-BSA counts in 1.0 g of perfusate. n equals number of lungs studied. Control refers to lungs to which only PMA was added (no neutrophils).

degranulation. Neutrophils not stimulated by PMA, like neutrophils treated with anti-Mo1 antibody, remained round and did not undergo spreading on or injure the vascular endothelium (Fig 3, column A). As a specificity control for the effect of anti-Mo1 antibody, in parallel experiments neutrophils were incubated with an isotype-identical murine monoclonal antibody (anti-Mo5).* Anti-Mo5 antibody failed to prevent neutrophil sequestration in the lung (Table 1, experiment C), or the PA pressure increase following the addition of the neutrophils to the perfusate (3 ± 0.6 mm Hg, n = 4, P = NS), or lung injury induced by the subsequent addition of PMA (Fig 2, column 5).

Effect of anti-Mo1 antibody on granule enzyme release. PMA stimulation of human neutrophils in the lung perfusate resulted in the secretion of the specific granule components lysozyme and lactoferrin into the lung perfusate (Fig 4). The addition of PMA in the absence of neutrophils resulted in no detectable increase in granule components. Incubation of human neutrophils with anti-Mo1 antibody had no significant effect on release of granule constituents into the lung perfusate following the addition of PMA. On the other hand, mere exposure of nonstimulated human neutrophils to rat lung endothelium led to the release of a small but significant (p < .001) amount of lactoferrin.

Effect of anti-Mo1 antibody on ferricytochrome c *reduction.* Stimulation of neutrophils with PMA caused a time-dependent reduction of ferricytochrome c in the lung perfusate (Fig 5). The average rate of ferricytochrome c reduction by untreated, anti-Mo1–, and anti-Mo5–treated neutrophils occurred at 30 nmol/min/10^7 cells during the first ten minutes following stimulation with PMA. There was no significant reduction of ferricytochrome c in perfusates containing either PMA or neutrophils alone. PMA-stimulated human neutrophils preincubated with either anti-Mo1 or anti-Mo5 antibody produced rates (P = NS) of ferricytochrome c reduction comparable to those observed with nontreated neutrophils.

Effect of extracellular ferricytochrome c *on PMA-stimulated H_2O_2 generation.* As shown in Table 2, ferricytochrome c totally inhibited H_2O_2 release from FMLP-stimulated cells. In contrast, the amount of H_2O_2 release detected from cells stimulated by PMA was only partially attenuated by the presence of ferricytochrome c.

DISCUSSION

Following stimulation, activated neutrophils are able to damage neighboring target tissues such as endothelial cells. This model has been broadly adopted to explain inflammatory vascular damage such as lung injury in the adult respiratory distress syndrome.[10] Because neutrophils are capable of releasing a broad spectrum of effector substances, neutrophil contact with vascular endothelium may be relevant to the pathogenesis of vascular injury and subsequent pulmonary edema. Previous studies have directed considerable attention toward the mechanisms by which neutrophils adhere to endothelium in vivo and in vitro. It has been observed that monoclonal antibodies directed against the Mo1 surface glycoprotein can inhibit neutrophil aggregation and their adhesion to substrates.[15] Employing cells from a patient with a congenital deficiency of adhesive glycoproteins, Harlan et al found that activated neutrophils from the patient failed to induce disruption of an endothelial monolayer or increase ^{125}I-albumin passage through the monolayers.[29] Activated control neutrophils caused a nonlytic disruption of the endothelial monolayers which was mediated by an oxygen radical–independent mechanism.[29] In contrast, Shasby et al demonstrated a neutrophil-mediated increased albumin passage across endothelial monolayers by an oxygen-dependent mechanism.[30] Since the cultured monolayers may not mimic the response of endothelium in vivo, we chose to study the effect of neutrophil activation on changes in permeability using an intact vasculature. We found that treatment of human neutrophils with anti-Mo1 monoclonal

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*Anti-Mo5 recognizes a neutrophil plasma membrane protein (Mo5) of unknown function.[24] Anti-Mo5 monoclonal antibody represents a good control for the effects of anti-Mo1, since by immunofluorescence analysis[24] the density of Mo5 is equivalent to that of Mo1 on the surface of unstimulated human neutrophils. Moreover, exposure of neutrophils to degranulating stimuli results in an increase in the surface expression of both Mo1 and Mo5[24] (Mo5 being somewhat less than Mo1).

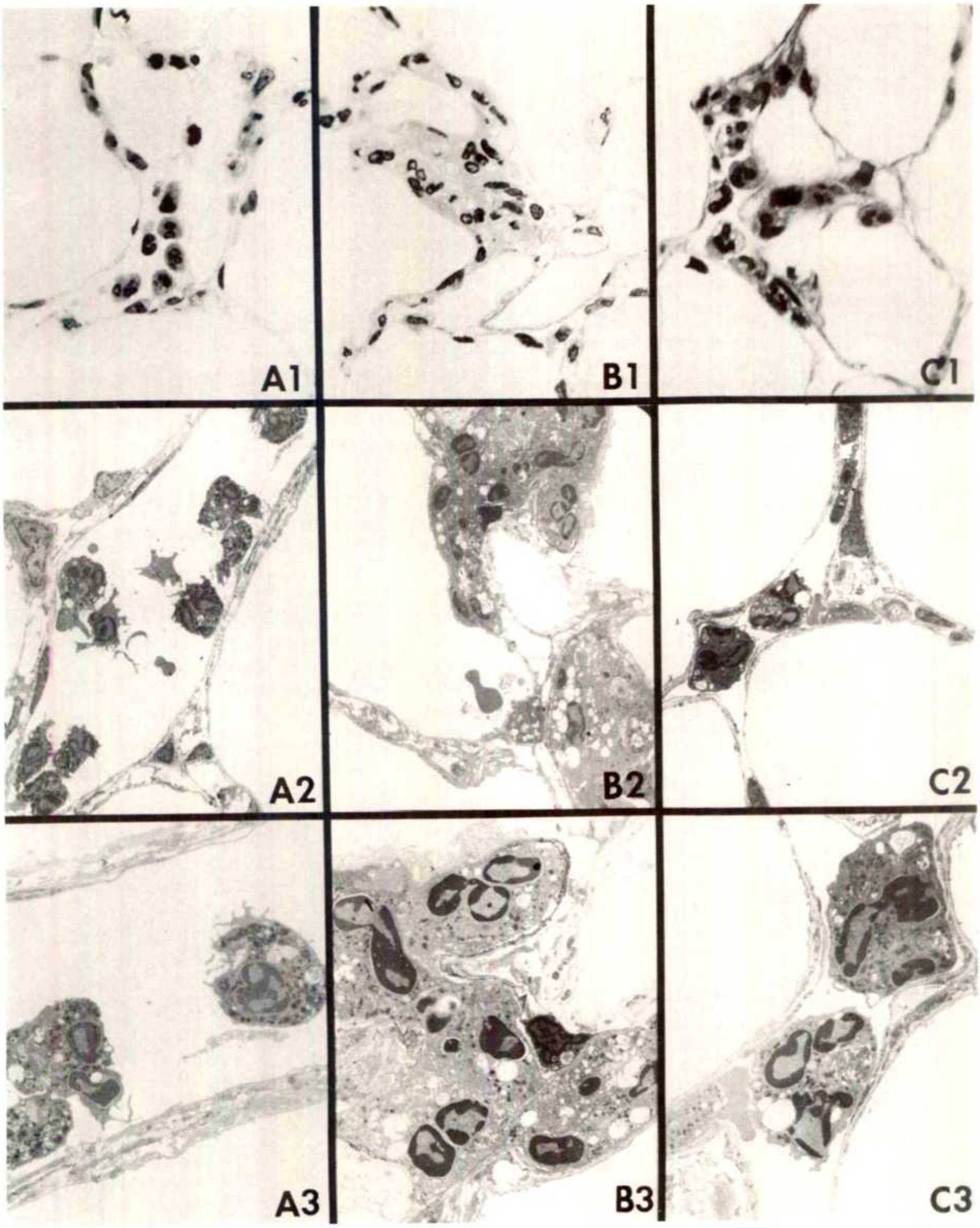

Fig 3. Photomicrographs of rat lungs exposed to human neutrophils. Line 1 original magnification 400×, current magnification; line 2 original magnification 2,600×, current magnification; line 3 original magnification 5600×, current magnification. Column A shows nonstimulated human neutrophils. Column B shows PMA-stimulated human neutrophils. Column C shows PMA-stimulated human neutrophils incubated with anti-Mo1 antibody. As seen in Column B, stimulation of PMNs by PMA resulted in the close approximation of the neutrophil to the endothelium and vacuolization consistent with degranulation. Gross endothelial cell injury is evident. Anti-Mo1-treated PMA-stimulated PMNs (Column C) or unstimulated PMNs (Column A) remained round and endothelial damage was not evident. Anti-Mo1 antibody did not prevent the development of PMN vacuolization. A contaminating red blood cell is seen in B2.

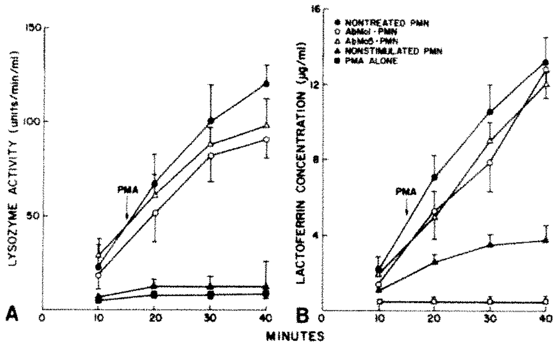

Fig 4. Release into lung perfusate of specific granule constituents (A) lysozyme and (B) lactoferrin over time. The time of addition of PMA is also shown (downward arrow). Nontreated PMNs (n = 5) refers to PMA-stimulated PMNs not incubated with a monoclonal antibody. AbMo1-PMN (n = 4) and AbMo5 (n = 4) refer to PMA-activated PMNs incubated with anti-Mo1 or anti-Mo5 antibody, respectively. Nonstimulated PMN (n = 4) refers to lung perfusates to which only PMNs were added. PMA alone refers to lung perfusates to which only PMA was added (no neutrophils added). There was a significant increase ($P < .05$) in lactoferrin over time for all groups except PMA alone. There was a smaller increase ($P < .05$) for nonstimulated PMNs compared to stimulated PMNs whether incubated or not incubated with monoclonal antibody. There was a significant increase ($P < .05$) in lysozyme release only for the three groups of neutrophils stimulated by PMA. The open squares in (B) refer to PMA alone.

antibody attenuated lung injury following exposure of neutrophils to PMA. The attenuation of the lung injury likely resulted from the inability of the neutrophils to undergo spreading and firmly attach to vascular endothelium. Simon et al[31,32] have demonstrated in epithelial cell cultures that incubation of PMA-stimulated neutrophils with Mo1 antibody inhibits cell injury. Although the Mo1 antibody did not prevent attachment of the neutrophils to the epithelial cells, it prevented neutrophil spreading and their close approximation to the target cells.

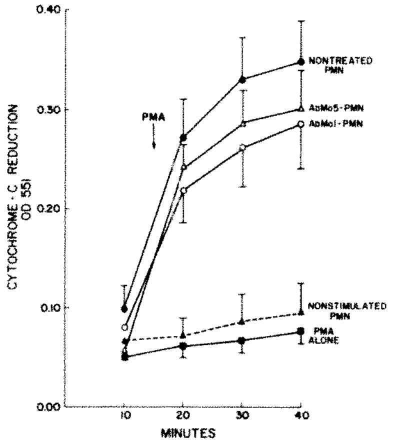

Fig 5. Reduction of ferricytochrome *c* only occurred in PMA-activated neutrophils whether or not they were incubated with a monoclonal antibody. There was no difference (P = NS) in the reduction of ferricytochrome *c* accompanying PMA-activated nontreated PMNs or PMNs incubated with anti-Mo1 antibody or anti-Mo5 antibody.

Table 2. Effect of Extracellular Ferricytochrome C on PMA- and FMLP-Stimulated H_2O_2 Generation

Experimental Conditions	H_2O_2 Generation (nmol H_2O_2/15 Min/5 × 10^6 Cells)	Percent Inhibition
PMN, FMLP	14.2 ± 1.2	
PMN, ferricytochrome *c*, FMLP	0	100%
PMN, PMA	123.6 ± 6.4	
PMN, ferricytochrome *c*, PMA	84.2 ± 5.4	32%

Experiments were performed on isolated human neutrophils in a test tube system containing 5 × 10^6 cells/mL and 50 µmol/L ferricytochrome *c*.

We demonstrated that treatment with anti-Mo1 monoclonal antibody did not prevent the PMA neutrophil–induced lung injury by inhibiting the production of potentially noxious substances such as oxidants and granule proteases. This is consistent with in vitro studies that have shown that anti-Mo1 antibody does not inhibit degranulation or superoxide production.[23] Finally, inhibition of lung injury did not arise from a nonspecific effect of a murine IgG2a antibody, since anti-Mo5, an IgG2a antibody that recognizes another neutrophil membrane constituent,[24] failed to attenuate lung injury.

One of the apparent advantages of our isolated perfused rat lung–human neutrophil system is the ability to quantify neutrophil activation in situ by monitoring both the generation of oxidants and the release of specific granule constituents into the perfusate. We found that the presence of ferricytochrome *c* in our lung perfusate did not quantitatively alter acute pulmonary injury of PMA-activated neutrophils as compared to those experiments carried out in the absence of ferricytochrome *c* (data not shown). A possible explanation for the lack of inhibition of injury by ferricytochrome *c* may be explained by the ability of PMA to initiate vesicles within intact neutrophils that are sequestered from the direct inhibitory effect of the superoxide scavenger. Other investigators have shown that during contact of human neutrophils with opsonized particles, the presence of ferricytochrome *c* failed to completely abolish H_2O_2 release from the cells. In contrast, there was no detectable H_2O_2 release from neutrophils pretreated with cytochalasin B to prevent phagosome formation in the presence of ferricytochrome *c*. These data suggest that the formation of "privileged sites" within the phagosome that are inaccessible to ferricytochrome *c* can lead to the generation of diffusable H_2O_2. In our model, ferricytochrome *c* which was used as an extracellular indicator of superoxide production likely did not scavenge superoxide formed within the neutrophil. Cytochemical studies previously have demonstrated that PMA induces formation of cytoplasmic vesicles and channels in neutrophils which exhibit H_2O_2 staining.[33] It is likely that PMA stimulation of the human neutrophils in our lung model induced similar vesicles hidden from the scavenging effect of ferricytochrome *c*. Indeed, we showed that the rate of H_2O_2 formation by PMA-stimulated human neutrophils in vitro was not completely abolished in the presence of extracellular ferricytochrome *c*, indicating that a substantial amount of H_2O_2 produced during PMA stimulation escaped the scavenging effects of ferricytochrome *c*. In contrast, neutrophils activated by FMLP, which leads to total release of superoxide to the extracellular environment, were inhibited from generating H_2O_2 by the presence of ferricytochrome *c* in the supernatant.

We found that pretreatment of neutrophils with anti-Mo1 antibody failed to completely inhibit sequestration of unstimulated neutrophils or cells exposed to PMA. The detection of residual soluble anti-Mo1 monoclonal antibody in lung perfusate even after PMA administration (which has previously been shown to induce an increase in surface Mo1 expression by neutrophils[34]) indicates that the concentration of anti-Mo1 antibody used in these experiments was far in excess of that required to saturate all neutrophil Mo1 antigenic sites. Another possible explanation of the failure to completely prevent cell adherence may relate to the existence of other adherence-promoting leukocyte surface antigens besides that recognized by our anti-Mo1 reagent. In support of this possibility are studies of Harlan, Beatty, Wallis, and their colleagues[18,19,35] in which a monoclonal antibody (60.3[36]) directed against the 95-kD β chain that is common to Mo1 and two other surface heterodimeric glycoproteins (LFA-1 and pl50,95) resulted in a significant inhibition of the adhesion of neutrophils[18,19] and monocytes[35] to endothelial surfaces. Whether the structurally related glycoproteins LFA-1 and p150,95 are also involved in neutrophil adhesion[37] is a subject for further study.

In our studies, we observed that the exposure of neutrophils to rat endothelium resulted in the release of a small but significant amount of the specific granule constituent lactoferrin. These observations are similar to reports by other investigators who have found that attachment of neutrophils to surfaces leads to exocytosis of specific granule constituents.[38] Despite the release of granule constituents, this was insufficient to lead to detectable lung injury in our system. Similarly, PMA by itself or by stimulating cells present in the lung did not lead to the intravascular generation of superoxide or lung injury. This is in contrast to the report by Dyer[39] demonstrating a neutrophil independent effect of PMA on lung injury in sheep.

In conclusion, PMA-activated human neutrophils in isolated perfused rat lungs induced acute edematous injury which could be markedly attenuated by incubation of the neutrophils with anti-Mo1 antibody. The extent of lung injury paralleled that observed in isolated rat lungs utilizing a homologous system employing rat neutrophils and rat plasma activated by the complement activator cobra venom factor.[25] In this homologous system lung injury was also neutrophil-dependent, since the activation of complement by cobra venom factor in plasma-perfused lungs in the absence of neutrophils did not result in lung injury. Anti-Mo1 antibody did not inhibit the release of either superoxide or granule constituents but led to an increase in the number of circulating cells in the perfusate, suggesting that the attenuation of lung injury by the monoclonal antibody resulted from interference of neutrophil adhesion to the endothelial cell surface by the specific monoclonal antibody. These studies indicate the necessity of intimate neutrophil–endothelial cell contact for the initiation of target cell injury.

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Increased Susceptibility of Peripheral Mononuclear Cells of Leukemic Patients to HTLV-I Infection In Vitro

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Peripheral mononuclear cells (MNC) collected from 12 healthy donors and 44 leukemic patients at various stages of the disease were tested for natural killer (NK) activity and for their susceptibility to HTLV-I infection in vitro, measured in terms of percentage of p19 positive cells. MNC from leukemic donors at any stage of leukemia (ie, onset or relapse, ON/REL; complete remission or off-therapy, CR/OT donors) were highly susceptible to HTLV-I infection. This was true for acute leukemias of lymphoblastic (ALL) or nonlymphoblastic (ANLL) type. MNC of ON/REL patients were more susceptible to HTLV-I than those of CR/OT donors. In addition, leukemic blasts were more rapidly infected (ie, within five to seven days) than the HTLV-I–susceptible normal cord-blood lymphocytes. However, the presence of circulating blasts was not essential to virus susceptibility, since CR/OT MNC, presumably free of leukemic blasts, were still more susceptible to HTLV-I than normal cells. Basal NK function of MNC from leukemic patients was significantly lower than that detectable in healthy controls. However, no correlation was found between susceptibility to HTLV-I infection and NK activity.
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HUMAN RETROVIRUSES of different HTLV groups have been isolated from patients affected by certain T cell malignancies in various geographic areas, and have been implicated in the development of the disease.[1-9] The HTLV-I subgroup was found to be able to infect T lymphocytes in mixed cultures in vitro between virus-producer donor cells and susceptible target lymphocytes, especially when umbilical cord blood[10,11] or bone marrow[12] lymphocytes were used as target cells. Adult T lymphocytes appear to be more resistant to infection than relatively immature target cells, as evidenced by studies on the expression of the HTLV-I structural protein p19 on target cell membrane, and DNA blot hybridization to detect the HTLV-I provirus integrated in the host cell genome.[13]

In order to study the host cell range and the regulation of HTLV-I infection, we have investigated whether the relatively immature lymphocytes present in the peripheral mononuclear cells (MNC) of leukemic patients would be highly susceptible to in vitro infection with the virus. The results of the present investigation confirmed this hypothesis and provided additional evidence that peripheral blood lymphocytes of patients in complete remission (ie, without evidence of circulating leukemic blasts) are still more susceptible to HTLV-I infection than normal T lymphocytes of adult donors.

MATERIALS AND METHODS

Cell lines. The human erythroleukemia K562[14] cell line was used as a target for the NK activity of effector lymphocytes in a four-hour ^{51}Cr release assay. MT-2, a HTLV-I producer cell line, was obtained by in vitro coculture of normal human cord-blood lymphocytes and adult T cell leukemia cells (ATL) positive for HTLV-I.[15] MOLT-4 is a T cell line obtained from peripheral blood cells of a young patient with acute lymphoblastic leukemia (ALL).[16] These cell lines were maintained as stationary suspension cultures in culture medium RPMI 1640 supplemented with 20% fetal calf serum (FCS) and 2 mmol/L glutamine (GIBCO, Grand Island, NY) plus 50 μg/ml of gentamycin (hereafter referred to as complete medium [CM]).

Leukemic patients. Peripheral mononuclear cells (MNC) were collected from 44 leukemic patients, including 27 adults (15 male and 12 female patients) and 17 children (10 male and seven female donors). The age range for adults was between 16 and 62 years, and that for children was between 3 and 14 years.

The diagnosis was ALL in 37 patients, being one of B, nine of T, and 27 of non-T, non-B phenotype. The remaining seven patients had acute nonlymphoblastic leukemia (ANLL). The number of patients at different stages of the disease is illustrated in Table 1. MNC were collected from off-therapy patients at least one year after the end of maintenance therapy, from patients in complete remission during maintenance therapy, and from patients at the onset or relapse of the disease, before starting induction therapy. In addition, in three cases the samples were collected from the same donor in two different stages, ie, in remission and in relapse, 2 to 3 months later. Chemotherapy regimens used for remission induction and for the maintenance of remission include the use of vincristine, daunorubicin, L-asparaginase, adriamycin, prednisone for induction therapy, and methotrexate, vincristine, and cytarabine for maintenance therapy as previously described.[17]

Cocultures. Recipient MNC were separated from peripheral blood by centrifugation on Ficoll-Hypaque gradient. Virus producer MT-2 cells (2×10^6) were lethally irradiated (12,000 R) and cocultured with recipient MNC (10^7) in 25-cm^2 flasks in CM (5 mL final volume) containing 10% of T cell growth factor (TCGF or IL-2).[10,18,19] The cultures were fed with fresh CM plus TCGF (Cellular Products, Buffalo, NY) twice a week or more frequently, depending on the growth rate. Control irradiated MT-2 cells were also cultured separately and no viable cells could be detected after three days of culture.

Irradiation of tumor cells in vitro. MT-2 tumor cells were irradiated in vitro using a ^{137}Cs irradiator (Gamma Cells 1000, model "A", AECL, Canada) delivering gamma rays at the rate of 1,000 rads/min, suspended in CM in 15-mL tubes at a concentration of 10^6 cells/mL. The cells were then washed twice and resuspended in the same medium.

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Table 1. Susceptibility to HTLV-I Infection of Target Peripheral Blood MNC Cocultivated In Vitro With HTLV-I Producer Cells

Donors of Target Cells*	Adult	Child	ALL†	ANLL‡	Total	p19-Positive	Samples	P§			
						Number	(%)	P1	P2	P3	P4
Healthy controls	7	5	—	—	12	0	0	<0.01	<0.01	—	—
Off Therapy (OT)	1	5	6	0	6	3	50.0	—	—	—	NS‖
Complete Remission (CR)	9	8	17	0	17	12	70.6	—	—	—	—
Onset + Relapse (On + Rel)	17	4	13	8	21	21	100.0	—	—	<0.05	—
Total leukemic	27	17	36	8	44	36	80.0	—	—	—	—

*Target cells were cocultivated with irradiated (12,000 R) HTLV-I producer MT-2 lymphoblasts (see Materials and Methods). Leukemic donors include all donors affected by leukemia, listed under OT(off-therapy 1 to 10 years), CR (complete remission, under maintenance therapy), and On + Rel (Onset + Relapse). Target cells of On + Rel donors contained various percentages of blasts.

†ALL, acute lymphoblastic leukemia.

‡ANLL, acute nonlymphocytic leukemia.

§*P*, probability calculated according to chi-square analysis, comparing the percentage of p19-positive samples as follows: *P*1, healthy controls *v* leukemic; *P*2, healthy controls *v* OT + CR; *P*3, OT + CR *v* On + Rel; *P*4, OT *v* CR.

‖NS, not significant.

Assay for HTLV-I infection. Infection with HTLV-I, inducing p19 protein expression in host cells, was determined by calculation of the percentages of p19 positive leukocytes. Fixed cells were analyzed by indirect immunofluorescence for the presence of HTLV-I p19 internal virus component using a murine monoclonal antibody specific for the purified p19 protein, and rabbit antimouse F(ab)2 IgG conjugated with fluorescein isothiocyanate (FITC, Cappel, Cochranville, PA) as previously described.[20]

Surface phenotype analysis of cells by monoclonal antibodies. Identification of cells positive for antigens reactive with different monoclonal antibodies and conventional surface markers (E rosette and sIg) were carried out as previously described in detail.[21,22] Murine monoclonal antisera specific for subsets of human T lymphocytes,[23,24] eg, T cells (OKT3), E rosette receptors (OKT11), the helper/inducer T cells (OKT4), cytotoxic/suppressor T cells (OKT8), immature hematopoietic stem cells (OKT10), tranferrin receptor (OKT9), histocompatibility locus D antigens (HLA-DR)[25] and antigen expressed on monocytes (OKM 1)[26] were used to label live cells. Positive cells were identified using FITC-labeled goat antimurine IgG F(ab)2-specific serum (Kallestad, Delta Biologicals, Rome). OKT reagents were purchased from Ortho Pharmaceutical Corp. (Raritan, NJ), and the other reagents from Becton Dickinson FACS Systems (Sunnyvale, CA).

Terminal deoxynucleotidyl transferase. A cytocentrifuge preparation of peripheral blood cells was fixed in methanol for 30 minutes, washed in PBS for 10 minutes, and analyzed for terminal deoxynucleotidyl transferase (TdT) utilizing rabbig IgG antihuman TdT and, subsequently, FITC-labeled goat antirabbit IgG (Bethesda Research Laboratories, Bethesda, MD).

Blast purification on Percoll gradients. Blast purification on Percoll gradients was performed as described by Hakos.[27] Briefly, MNC obtained after separation on Ficoll-Hypaque gradients were further layered on two Percoll gradients. Percoll was diluted with RPMI 1640 plus 50 μg/mL of gentamycin to concentrations of 40% (1.06 g/mL) and 36% (1.05 g/mL); then 5 mL of 40% Percoll was placed at the bottom of a 15-mL tube (Falcon, Oxnard, CA) followed by 5 mL of 36% Percoll over which MNC were stratified. Tubes were centrifuged at 550 g for 30 minutes, and blast cells were concentrated at the 36% interface, while small mature lymphocytes were placed at the bottom of the tube.

DNA preparation. High molecular weight DNA was extracted by proteinase K digestion followed by organic solvent extraction, according to standard procedure.[28]

Dot blot analysis. 3 μg of DNA from each sample were denatured just before spotting by incubation in 0.2 mol/L NaOH for 10 minutes at 65°C. The samples were then neutralized by adding 1 vol of 2 mol/L NH_4 acetate. The DNA samples were spotted on a nitrocellulose filter (Schleicher and Schüll, Dassel, FRG) previously saturated in 1 mol/L NH acetate. The filter was dried and then baked for two hours at 80°C. Prehybridization was performed for three hours at 65°C on a shaking platform in 4× SET buffer (NaCl 3 mol/L, TRIS 0.06 mol/L, EDTA 40 mmol/L), and 10× Denhart (polyvinylpirrolidone, bovine serum albumin, and Ficoll), 0.1% sodium dodecyl sulfate (SDS) (Sigma, St Louis), and 100 μg/mL salmon sperm. The filters were then hybridized in the same solution with a ^{32}P nick-translated HTLV-I probe (3×10^6 cpm/mL, specific activity 2×10^8 cpm/μg). The HTLV-I probe is a Sst I subclone from a MT-2 genomic clone containing the complete virus.[29] The filters were washed in a solution of 10× Denhart, 4× SET, and 0.1% SDS for 30 minutes and successively in 2× SET and 0.1% SDS for 30 minutes, both on a shaking platform at 65°C. The filters were exposed to X ray film with an intensifying screen ("X-Omatic," Kodak, Rochester, NY) for 66 hours.

Cytotoxicity assay. The NK activity was determined using a ^{51}Cr release assay, as previously described.[30] Briefly, graded concentrations of effector cells (E) were suspended in CM containing 25 mmol/L HEPES buffer, mixed with 2×10^3 ^{51}Cr-labeled target cells (T) in round-bottom 96-well microtiter plates (Greiner C.A. and Söhne, Nurtingen, FRG) (final volume 0.2 mL) and incubated for four hours in a 5% CO_2 incubator. At the end of the incubation period the plates were centrifuged (800 g for 10 minutes) and the radioactivity of 0.1 mL of the supernatant was measured in a γ-scintillation counter (Packard Instrument, Downers Grove, IL). The baseline ^{51}Cr release was that released spontaneously from target cells and in no case did it exceed 10% of the total radioactivity incorporated by target cells. Experimental results were expressed as the percentage of specific lysis over the spontaneous release and were calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{cpm(t)} - \text{cpm(s)}}{\text{cpm(T)}} \times 100$$

where cpm(t) is the mean cpm released in the presence of effector cells, cpm(s) is the mean cpm released spontaneously by target cells, and cpm(T) is the total amount of ^{51}Cr incorporated into target cells.

Calculation of lytic units. Dose–response curves were obtained by plotting the percentage of specific ^{51}Cr-release and the effector–target (E/T) ratios. The best-fit curve for this function was found to be logarithmic in accordance with previous reports.[31] A lytic unit (LU10) was defined as the number of effector cells extrapolated from the dose–response curve which was required to achieve n% specific target cell lysis. The amount of LU10/10^6 effector cells was calculated by dividing 10^6 by the number of lymphocytes corresponding to 1 LU10. The cytolytic activity was considered low when it was less than 1 LU10/10^6 cells. The mean ± standard deviation (SD) of LU10/10^6 cells of normal lymphocytes preparation was found to be 26.4 ± 12.7. Therefore, low-activity samples were considered those below the mean minus 2 SD (ie, 1 LU10/10^6 MNC).

RESULTS

Peripheral MNC obtained from healthy donors and leukemic patients at various stages of the disease were cocultured with irradiated infecting MT-2 cells and tested for p19 expression at weekly intervals for 5 weeks. The results illustrated in Table 1 show that MNC of leukemic patients were particularly susceptible to HTLV-I infection, as evidenced by the significant percentage of samples which became p19-positive within 1 to 5 weeks of coculture (Table 1, *P*1). The increased susceptibility to HTLV-I infection with respect to that of normal MNC was particularly evident in MNC collected from donors at the onset or relapse (On + Rel) stage of the disease, which is associated with the presence of blast cells in peripheral blood and bone marrow. This was true for ALL or ANLL. In the latter instance the number of T lymphocytes, targets of the virus infection,[9-11,15] was very limited, being the leukemia cell population largely represented by myeloblasts.

The data in Table 1 show also that putatively normal lymphocytes obtained from patients off-therapy (OT) and those in complete remission (CR) were significantly more susceptible to HTLV-I infection than normal adult MNC (Table 1, *P*2), whereas no difference in virus susceptibility was found between MNC of OT and CR donors (Table 1, *P*4). However, the statistical analysis of the percentage of p19-positive samples indicates that MNC of On + Rel donors were more susceptible to HTLV-I infection that those collected from OT + CR patients (Table 1, *P*3).

These findings were further supported by the kinetic analysis of virus infection illustrated in Fig 1, showing that MNC containing blast cells (of On + Rel donors) were more rapidly converted to p19-positive cells following exposure to MT-2 HTLV-I than MNC collected from OT + CR patients. Moreover, leukemic samples, especially those collected from On + Rel donors, were more readily infected than those of normal umbilical cord blood MNC (Fig 1).

The close association between p19 positivity and virus infection was confirmed by the finding that cells carrying the p19 antigen were also positive for HTLV-I provirus. This was evidenced by DNA dot blot analysis, using the DNA extracted from infected MNC of ALL and ANLL donors and a nick-translated ^{32}P-labeled probe containing almost the entire HTLV-I genome (Fig 2). Moreover, control cocultures were carried out mixing leukemic cells of an ANLL donor or of in vitro growing MOLT-4 cell line with irradiated Epstein-Barr virus (EBV)-infected human B lymphoblastoid cells (X_3O_3 line). In this case the cocultures remained consistently negative for the p19 antigen during up to 2 weeks of observation. On the other hand, the same target cells cocultivated with the irradiated MT-2 line showed high percentages of p19-positive cells as early as at four days of coculture (data not shown).

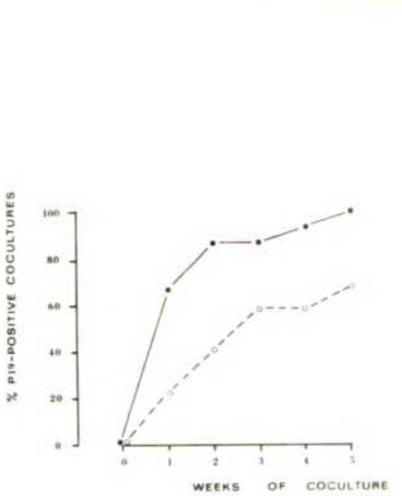

Fig 1. Time course of appearance of p19 positivity after HTLV-I infection of MNC obtained from On + Rel (●—●, 15 cases), or OT + CR (○—○, 15 cases) donors, cocultured with irradiated MT-2 cells. Chi-square analysis of the percentages of p19-positive cocultures (ie, showing more than 10% p19-positive cells) showed a significant difference between the two groups ($P < 0.05$) at 1, 2, 4, and 5 weeks postinfection. Eight different samples of cord-blood lymphocytes cocultured with HTLV-I donor cells at the same conditions showed no positivity after 2 weeks of coculture. Eighty percent of the samples were positive (ie, showing more than 10% p19-positive cells) at 3 weeks and 100% at 4 weeks of coculture.

Cell phenotype of T lymphocytes infected in vitro with HTLV-I was found to be predominantly of helper/inducer class, ie, positive for the surface antigen recognized by OKT4 monoclonal antibody and positive for HLA-DR determinants.[32] Similarly, the majority of the phenotype markers associated with infected MNC of the leukemic patients described in the present study were found to be reactive with OKT4 and anti-HLA-DR antibodies. This is shown in a typical example of surface phenotype patterns of infected MNC of ALL donors, as illustrated in Table 2. In this case blast cells containing 10% only of OKT4 positive lymphocytes yielded an infected line expressing predominantly the OKT4 phenotype. In addition "normal" lymphocytes of an OT donor infected by HTLV-I virus generated a cell line represented largely by OKT4 blast cells (Table 2). However, the data obtained in the present study pointed out also that pure ANLL blasts, deprived of T lymphocytes, were rapidly infected by the virus. Blast cells from peripheral blood obtained from ANLL donors were purified on Percoll gradients as described in Materials and Methods. The cells were cocultured with irradiated MT-2 donor cells and tested for

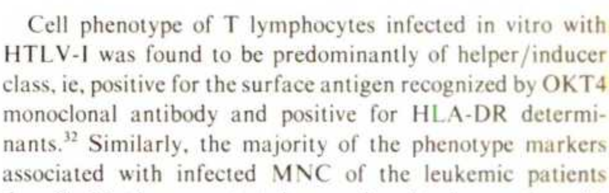

Fig 2. A representative experiment of dot blot analysis on genomic cellular DNA is shown. HTLV-I provirus is integrated in the DNA obtained, respectively, from two p19-positive cocultures (A and B) which survived more than 5 weeks. Cocultures were performed using recipient MNC bearing the phenotype of ANLL (onset) M5 variant (A), and of ALL (onset) T cell variant (B), respectively. Positive control (C) is DNA extracted from HTLV-I donor MT-2 cells. Negative control (D) is DNA extracted from the p19-negative erythroleukemia K562 cell line.

Table 2. Cell Surface Markers of MNC of an ALL Patient Before or After Infection With HTLV-I

Leukemic Cells		Blasts	Percent of Positive Cells							
Code	Type	%	p19	OKT3	OKT4	OKT8	OKT9	OKT11	Tdt	HLA-Dr
MT-2	H/CB	100	100	—	100	—	ND	45	ND	100
CC	B-ALL (ON)	90	0	30	10	4	50	5	+	0
CC/MT-2	H/B-ALL (ON)	90	90	70	90	5	82	70	—	100
CF	cALL (OT)	0	0	70	49	28	0.1	71	—	12
CF/MT2	H/cALL (OT)	90	85	39	93	—	ND	30	ND	100

Abbreviations: H/CB, HTLV-I infected cord blood cell line; B-ALL (ON), acute lymphoblastic leukemia (B cell variant); ON, onset; H/B-ALL (ON), HTLV-I–infected ALL, tested on day +54 of coculture with irradiated MT-2 cells; cALL (OT), acute lymphoblastic leukemia (common type); OT, off therapy; H/cALL (OT), HTLV-I–infected ALL tested on day +48 of coculture with irradiated MT-2 cells; ND, not done.

p19 positivity and phenotypic markers on days 5, 11 and 22 of coculture. The results (Table 3) show that blasts were HTLV-I–positive as early as five days after the coculture and retained their original surface markers. Actually no cell of the T lymphocyte lineage, reactive with "pan T," OKT3, or OKT11 antibodies could have been detected in this HTLV-I–positive line.

The studies concerning the basal NK function of MNC of various origins, measured before coculturing with the virus producer MT-2 cells, are illustrated in Tables 4 and 5. The results expressed in terms of geometric mean of LU10/10^6 MNC or as percentage of samples showing low NK activity (LNK, ie, less than 1 LU10/10^6 MNC, see Materials and Methods) reported in Table 2 show that: (*a*) NK activity was significantly lower in lymphocytes of leukemic patients than that detectable in healthy controls. Moreover, the percentage of LNK samples was higher in leukemic donors than that detectable in healthy controls; (*b*) the NK activity and percentage of LNK samples found among OT + CR patients did not reach a statistically significant difference from those detectable among donors bearing active disease (On + Rel patients); and (*c*) the percentage of LNK samples was significantly higher in MNC preparations.

DISCUSSION

Considerable experimental evidence is now available showing that HTLV-I can be transmitted in vitro to a variety of target cells, using cell-to-cell transfer in tissue culture.[10-13] There are a number of studies demonstrating that cocultivation of HTLV-I–producing cells with recipient cells is far more efficient for transmitting the retrovirus than is extracellular HTLV-I.[10] The data presently available suggest also that preferential target cells of the virus would be human T lymphocytes carrying the OKT4 marker. This appears to be especially true in the case of spontaneous HTLV-I–positive cells of patients with adult T cell leukemia–lymphoma[33] or of cord-blood lymphocytes infected by in vitro cocultivation with virus-donor cell lines.[32] However, when bone-marrow recipient cells were used for in vitro HTLV-I transmission, infected T cells were alternatively of OKT4+, or OKT8+ or OKT4−/OKT8− phenotypes.[12] The host range for HTLV-I infection appears to be even larger in selected experimental conditions, since B-cells[34] and cells of nonlymphoid origin,[35] including endothelial cells,[36] have been successfully infected with the virus. In any case, the pattern of HTLV-I transmission in vitro shows preferential susceptibility of lymphoid cells at relatively immature stages (eg, cord blood or bone marrow cells), whereas adult MNC appear to be more resistant to virus infection.[13,37]

Therefore, it was anticipated that mononuclear cells collected from patients with lymphoproliferative disorders would have been particularly susceptible to the infection with the retrovirus, especially when immature hemopoietic cells are present in the peripheral blood.

Preliminary data have shown that cultured human leukemic lines are highly susceptible to HTLV-I infection (P. Markham, personal communication, February 1985). The results of the present study confirm that circulating MNC, including blast cells of leukemic patients, are easily and rapidly infected with the virus. However, the increased susceptibility to HTLV-I infection of cells derived from leukemic donors was found not only in MNC of On/Rel patients but also in those collected from CR/OT donors. Actually, in the case of MNC of CR/OT origin, appreciable

Table 3. Time Course of Appearance of p19-Positive Cells and Expression of Cell Surface Markers of Purified ANLL Blasts* Cocultured With Irradiated MT-2 Cells

	Percentage of Positive Cells				
Day of Coculture	p19	OKT3	OKT11	LeuM1	HLA-DR
0†	0	0	0	20	86
5	24.6	0	2	17	100
11	38.8	0	0	18	100
22	30.0	0	1	57	49

*ANLL (M4 variant) blasts (Percoll fraction; see Materials and Methods) were also negative for LeuM2, S47, and My-7 surface markers. The pellet fraction of ANLL MNC contained 43% and 49% positive cells for OKT3 and HLA-DR markers, respectively.

†Day 0 data refer to ANLL blasts before coculture with HTLV-I donor MT-2 cells.

Table 4. Comparative Studies on the NK Activity of Fresh MNC Obtained From Healthy or Leukemic Donors, Assayed Before Coculture With MT-2 Cells

Group No.	Donor Type*	No.† Obs.	NK Activity‡ LU10/10^6 MNC GM (SE)	$P1$§	LNK‖	$P2$¶
1	Healthy controls	12	128 (76.7-213.5)	—	8.3	—
2	Leukemic controls	43	0.25 (0.11-0.55)	<0.01	72.1	<0.05
3	OT + CR	28	0.6 (0.2-1.6)	—	64.3	—
4	On + Rel	15	0.047 (0.013-0.17)	NS	86.7	NS
5	p19-Negative (total)	19	34.5 (16.4-72.9)	—	33.3	—
6	p19-Positive (total)	29	0.098 (0.036-0.26)	NS	75.9	<0.05
7	p19-Negative (leukemic)	7	4.4 (0.99-19.6)	—	50	—
8	p19-Positive (leukemic)	29	0.098 (0.036-0.26)	NS	75.9	NS

*MNC from donors of the type illustrated in the column. OT, off-therapy; CR, complete remission; On, onset; Rel, relapse.

†Number of observations.

‡Expressed as the geometric mean (GM), and GM − standard error (SE), and GM + SE in parentheses of LU10/10^6 MNC values.

§Probability calculated according to t test analysis, comparing NK activity between group 1 and 2, 3, and 4, 5, and 6, and 7 and 8. NS, not significant.

‖Percentage of samples with low NK activity (<1 LU10/10^6 MNC, see Materials and Methods) with respect to the total number of observations for each group.

¶Probability calculated according to chi-square analysis, comparing the same groups considered for $P1$ evaluation.

amounts of target immature lymphocytes do not appear to be present. Moreover, no increase of the proliferative response of CR/OT MNC in MNC/MT-2 coculture was found with respect to that detectable in normal MNC/MT-2 coculture (data not shown).

In any case, the present studies demonstrated that MNC containing blast cells (of On/Rel donors) are more rapidly converted to p19-positive cells following exposure to MT-2 HTLV-I than lymphocytes from CR/OT donors, as shown in Fig 1. This is consistent with previous studies showing that proviral integration is favored by target cell proliferation,[12] as expected to occur in leukemic blasts. That OKT4 cells were the primary target of virus infection seems to be confirmed by the findings described in Table 2, showing that infected HTLV-I lines were predominantly of OKT4 phenotype.

This occurred also in the case of B-cell leukemia containing a limited number of OKT4-positive lymphocytes. In this case, however, one cannot rule out the possibility that target leukemic B cell blasts could have been converted by HTLV-I infection to cells carrying T cell–like markers, including determinants cross-reactive with the OKT4 antigen.

Table 5. Relationship Between Time of HTLV-I Infection Expressed in Terms of Week(s) of the Appearance of p19-Positive Cells and Basal NK Function

Week of p19 Positivity*	No. of Observations	LU10/10^6 MNC GM (SE)†
1	14	0.140 (0.033-0.59)
2	6	0.048 (0.004-0.58)
3	4	7.900 (2.7-23.2)
5	3	0.036 (0.001-1.27)

*Time in week(s) of appearance of more than 10% p19-positive cells, after day 0 of coculture between recipient and virus-producing MT-2 lymphoma cells.

†In parentheses: mean − standard error (SE) and mean + SE. The Pearson r coefficient for the relationship between the week of p19 positivity and the levels of NK function expressed in terms of LU10/10^6 MNC is −0.123, showing that there is no relationship between the two parameters.

In the case of MNC collected from On/Rel of ANLL leukemic patients, the "preferential" immature T targets of HTLV-I did not appear to be present in the cocultures (Table 3) and yet rapid and total conversion to p19-positive cells occurred (Tables 1 and 3). This could be explained considering that early and successful in vitro transmission of HTLV-I could occur in target cells other than immature T lymphocytes and in particular blast cells of myeloid origin.

The possible role of NK function in virus transmission in vitro has been considered in the present study, as illustrated in Tables 2 and 3.

A number of reports in the literature suggest that natural immunity, at least in part represented by the NK system, would be involved in immune surveillance against the outgrowth of transformed cells and cancer progression.[38,39] The observation that NK activity of HTLV-I–resistant MNC of normal donors is greater than that of HTLV-I–susceptible MNC of leukemic subjects (Table 4) seems to support this hypothesis. However, in the case of HTLV-I–susceptible MNC obtained from leukemic patients at any stage of the disease, one would expect that MNC samples with low NK activity were more susceptible to HTLV-I infection, or more rapidly infected by the virus than those with high NK activity. Since this was not the case (Tables 4 and 5), it can be suggested that virus infection is not profoundly affected by the NK system, at least within MNC of leukemic donors, although the number of observations is too low to detect a limited influence of natural immunity on virus transmission. This possibility is also supported by the observation that the majority of the p19-positive transformed lines are scarcely susceptible to NK-mediated cytolysis (data not shown). As already mentioned, the majority of MNC preparations obtained from healthy donors were associated with high NK activity. Therefore it is reasonable to assume that additional properties, distinct from NK function, would be responsible for the relatively high HTLV-I resistance found in normal adult lymphocytes.

In conclusion, the results obtained in the present study show that: (*a*) leukemic MNC are more susceptible to HTLV-I infection than normal lymphocytes; (*b*) morphologically normal lymphocytes of leukemic patients, off-therapy or in complete remission, are more susceptible to HTLV-I than T cells of healthy donors; and (*c*) no convincing evidence was found that the level of basal NK activity markedly affects the susceptibility of leukemic MNC to viral infection.

The reason for this is not presently known. It might well be a consequence of altered interaction among cellular genes that follows the drug therapy and/or the leukemic transformation.

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Membrane Receptors for Interleukin 2 on Hematopoietic Precursors in Chronic Myeloid Leukemia

By Giuseppe Visani, Ruud Delwel, Ivo Touw, Frederik Bot, and Bob Löwenberg

In this report we present data on the expression of IL2 receptors on chronic phase CML cells. Using an anti IL2 receptor monoclonal antibody (McAb aIL2r) in indirect immunofluorescence we found significant proportions (42.2% ± 19.7 SD) of the CML cells (previously depleted of E rosetting T cells) to be IL2 receptor positive following incubation in suspension for 18 hours at 37°C. Noninduced cells did not express IL2 receptors. After induction the aIL2r positive and negative cell subpopulations were sorted and analyzed separately for morphology, lineage specific cell surface markers, and clonogenic cell numbers. The IL2 receptor positive CML subpopulations mainly contained blast cells and monocytes and revealed reactivity with myeloid McAbs but not with T cell, B cell, platelet, or erythroid markers. Clonogenic cells (CFU-GEMM, BFUe, and CFU-GM) were selectively recovered from aIL2r positive CML cells and thus were IL2 receptor positive. The addition of recombinant IL2 (rIL2) to CFU-GM and BFUe cultures, in concentrations from 50 to 500 U/mL, did not influence the efficiency of colony formation. Binding of a radiolabeled IL2 preparation to the in vitro activated CML cells indicated the presence of low affinity receptors for IL2. In contrast to CML, normal human marrow cells were consistently aIL2r nonreactive. Thus, IL2 receptor inducibility is a characteristic feature of CML clonogenic cells, which they share with AML, but not with normal marrow progenitors. The role of IL2 receptors in the regulation of proliferation of CML cells requires further investigation.

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INTERLEUKIN 2 (IL2) is a growth factor of T and B lymphocytes.[1-3] The expression of IL2 receptors on the surface of lymphocytic cells is a prerequisite for these cells to proliferate in response to IL2.[2,4] Recently it has been shown that receptors for IL2 can be induced on human monocytes[5,6] and acute myeloid leukemia (AML) cells[7] as well, following incubation in vitro.[6,7] Although the findings did not clarify the role of IL2 receptors in AML growth, the data suggested that these receptors may function to modulate the proliferation of human AML.

In the present studies we explored the possible presence and function of IL2 receptors in chronic myeloid leukemia (CML). We show that large proportions of CML cells from six patients, initially IL2 receptor negative, express the IL2 receptors after activation in culture. The IL2 receptor positive CML fraction contained the greatest part of the clonogenic cells, ie, CFU-GM, BFUe, and CFU-GEMM.

In contrast, normal bone marrow, including the colony forming progenitors, when treated and analyzed similarly, were consistently IL2 receptor negative. The addition of IL2 to the cultures, in doses ranging from 50 to 500 U/mL, did not modify CML colony growth and neither did blocking the IL2 receptors with an aIL2r McAb. Moreover, binding (that would have been significant for high affinity receptor presence) could not be measured with radiolabeled IL2, suggesting that most of the IL2 receptors induced on the surface of the CML cells exhibit a low affinity to the ligand.

MATERIALS AND METHODS

CML and normal bone marrow cells. CML cells were obtained from bone marrow (aspirated from the iliac spine) or peripheral blood of six patients in the first chronic phase of the disease following informed consent. The cells were separated through Ficoll-Isopaque, cryopreserved (7.5% DMSO and 20% fetal calf serum) and stored in liquid nitrogen, according to standard procedures.[8] After thawing, residual T lymphocytes were removed from the suspension with E rosette sedimentation and thus reduced to less than 1% of the nucleated cells, as previously described.[9,10] Normal marrow was obtained following informed consent from volunteers or patients without hematological disorders and prepared, and T cell depleted exactly as described for CML cells.

McAbs. The McAbs used in this study for the analysis of surface antigens are listed in Table 1.

Indirect immunofluorescence and fluorescence activated cell sorting. Cell surface membrane antigens were assayed by staining with monoclonal antibodies and goat antimouse immunoglobulins coupled to fluorescein isothiocyanate (GAM/FITC, Nordic, Tilburg, The Netherlands) as described.[16,17] The cells were then analyzed by fluorescence microscopy and by flow cytometry with a fluorescence activated cell sorter (FACS 440, Becton Dickinson, Mountain View, CA).[18]

Induction of the IL2 receptors. For induction of the IL2 receptors on the cell membrane, cells were incubated in suspension for 18 hours in the presence of phytohemagglutinin (PHA) (1% vol/vol, reagent grade; Wellcome, Dartford, England), 12-0-tetradecanoylphorbol-13-acetate (TPA) (10 μg/mL) (Sigma, St Louis) or without these additions. Subsequently, the cells were washed twice with phosphate buffered saline (PBS) and prepared for indirect immunofluorescence with the aIL2r McAb. Incubations using a McAb of the same immunoglobulin class (IgG1) (ie, OKT6 = CD1) were always included as controls. In some experiments anti IL2 receptor McAb (IgG1) + GAM/FITC stained cells were sorted and then analyzed for the expression of other surface antigens. In these experiments the sorted cells were stained with McAbs (not of the IgG1 subclass) + goat antimouse immunoglobulins, specifically reactive with the Ig class or subclass of this second antibody and coupled to the second fluorescent label tetramethylrhodamine isothiocyanate (GAM/TRITC). Analysis with McAbs of the IgG1 subclass were performed with cells previously labeled with anti-TAC McAb (IgG2a) instead

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Table 1. Specifications of McAbs

McAb	Isotype	Titres	Reactivity	Reference or Source
B 4.3 (CD15)	IgM	1:500	Metamyelocytes → granulocytes	11
S 4.7	IgM	1:500	Myeloblasts → granulocytes, monocytes, myeloid leukemias	12
S 3-13	IgM	1:50	CFU-GM, myeloblasts, monocytes, T lymphocytes, myeloid leukemias	12
Vim2	IgM	1:100	Granulocytes, monocytes, 90% myeloid leukemias	13
IV B5	IgG1	1:500	Erythrocytes (glycophorin A)	14
B4 (CD19)	IgG1	1:10	B cells	Coulter Immunology (Hialeah, FL)
C 17	IgG1	1:500	Platelets (glycoprotein IIIA)	14
VilA1 (CD10)	IgM	1:100	Common ALL antigen (CALLA)	15
T3 (CD3)	IgG2a	1:40	Mature thymocytes and T lymphocytes	Ortho Pharmaceutical (Raritan, NJ)
T6 (CD1)	IgG1	1:40	Common thymocytes, epidermal Langerhans cells	Ortho Pharmaceutical (Raritan, NJ)
aIL2r (CD25)	IgG1	1:20	Anti IL2 receptor	Becton Dickinson (Mountain View, CA)
antiTac (CD25)	IgG2a	1:1,000	Anti IL2 receptor	3

of aIL2r. Cells were then evaluated for surface membrane fluorescence using a fluorescence microscope. Sorting of the cells into IL2 receptor positive and negative fractions was carried out according to previously described techniques.[18]

Myeloid colonies. CML (0.5 × 10^5 cells) or normal bone marrow (1 × 10^5) cells were plated in 35-mm Petri dishes in 0.2 mL of semisolid medium on top of a 1-mL agar (0.5%) underlayer supplemented with human placenta conditioned medium (HPCM) (30% vol/vol).[19,20] Colonies of more than 50 cells were scored on day 12 of culture. Colony counts represent the mean estimates of triplicate cultures. Colonies were collected from the dishes and examined morphologically and immunologically according to established procedures.[16]

Erythroid colonies. Erythroid colonies (BFUe) were assayed by plating 0.125 × 10^5 CML cells or 0.25 × 10^5 normal marrow cells in 0.25-mL methylcellulose cultures containing Iscove's modified Dulbecco medium supplemented with bovine serum albumin (BSA), transferrin, lecithin, natrium selenite, betamercaptoethanol, 30% fetal calf serum, and human erythropoietin derived from the urine of a patient with aplastic anemia, as described.[21] Cells (0.125 × 10^5 CML cells; 0.25 × 10^5 normal marrow) were plated in cluster dish wells and kept at 37°C and 5% CO_2 in humidified air. Cultures were performed in quadruplicate; colonies (more than 50 cells) were counted on day 14.

Mixed colonies. Mixed colonies (CFU-GEMM) were grown as described by Fauser and Messner[22] with slight modifications.[18] Colonies were scored at day 15. Mixed colonies defined as containing red hemoglobinized cells plus at least 5% nonhemoglobinized cells were always verified cytologically.

Analysis of the effects of IL2 on CFU-GM and BFUe growth. In certain experiments modifications of the standard HPCM and BFUe systems were used. HPCM cultures were performed in triplicate in 24-well dishes (Greiner, Alphen aan de Rijn, the Netherlands). Cells (0.20 × 10^5) were plated in a 100-μL semisolid upper layer on the top of a 0.25-mL underlayer. The effects of IL2 were then tested by adding IL2 to the culture upper layers (500 units/mL). Simultaneous cultures were performed to which aIL2r (2.5 μg/mL); aIL2r + IL2 (500 U/mL) or the control McAb T6(CD1; IgG1) (2.5 μg/mL) had been added. Colonies were scored on day 12. Similar experiments were carried out to examine the effects of IL2 on BFUe growth.

Binding of radiolabeled IL2. To assess the binding properties of the IL2 receptors expressed by the CML cells, we performed affinity studies with radiolabeled ^{125}I-IL2 as previously described.[23] One million cells in 50 μL alpha medium with 10% BSA were incubated with serial concentrations of pure recombinant ^{125}I-IL2 (Amersham, UK) to a final concentration of 100 pmol/L. The cells were incubated with the radiolabeled IL2 for one hour at 37°C and then radioactivity was counted in a 4/200 automatic gamma counter (Micromedic, Horsham, PA). To correct for nonspecific binding of the ^{125}I-IL2, incubations were also performed in the presence of excess nonradioactive rIL2. All incubations were carried out in duplicate.

RESULTS

Induction of IL2 membrane receptors on CML cells. Fresh CML cells (T lymphocyte depleted) did not express IL2 receptors on their surface, as indicated by the negative immunofluorescence after labeling of the cells with the McAb aIL2r. On the other hand, high reactivities of the cells with the McAb aIL2r were apparent in all six cases following incubation of the cells in suspension (18 hours) (Fig 1, Table 2). The fraction of aIL2r positive cells was estimated at 42.2 ± 19.7% (mean ± SD; range 20% to 80%; n = 6). The induced cells did not show any reactivity with a control McAb of the same isotype (ie, OKT6; IgG1). IL2 receptor positive CML cells were separated by fluorescence activated cell sorting and examined morphologically and immunologically. The IL2 receptor positive cell fraction contained mainly Sudan black positive myeloblasts (Table 2) and monocytes, and expressed the myeloid antigens reactive with the McAbs S4-7 (37%), S3-13 (8%), and Vim2 (12%). The IL2 receptor positive subset showed no reactivity with reagents recognizing the lymphocytic T3 antigen, E rosette receptor, CALLA (CD10) and B cell antigens (B4; CD19), erythrocytic antigen glycophorin A and platelet antigen glycoprotein IIIA.

In contrast to these findings with CML cells, we were consistently unable to demonstrate any expression of IL2 receptors on T cell depleted normal marrow cells (n = 14 cases), before or after incubation (Fig 1).

Colony formation by IL2 receptor expressing CML cells. Activated CML cells were sorted into IL2 receptor

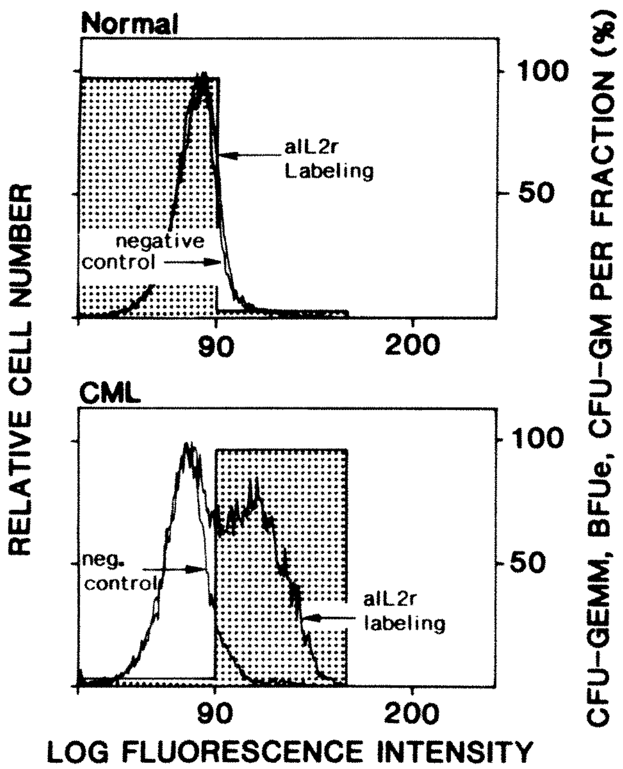

Fig 1. Fluorescence histograms indicating the IL2 membrane receptor expression on induced CML and normal bone marrow. As controls, cells were incubated with the fluorescent label but without IL2 receptor reactive McAb to assess background (negative) fluorescence. Colony-forming cells (CFU-GM, BFUe, and CFU-GEMM) were determined by sorting the cells into negative (channels 0 to 90) and positive (channels 91 to 255) fractions and the distributions are also indicated (stippled block diagrams). Two examples are given: (A) normal bone marrow (T cell depleted); and (B) CML bone marrow (T cell depleted).

positive and IL2 receptor negative fractions in three experiments and cultured for CFU-GM, BFUe, and CFU-GEMM (Table 3, Fig 1). The aIL2r positive fraction contained the majority of CFU-GM, BFUe, and CFU-GEMM. To exclude the possibility that IL2 receptor negative cells had been miscarried with the IL2 receptor positive sorts and had been responsible for colony formation, a control sort was carried out. For this purpose, the cells were stained with GAM/FITC alone and sorted on fluorescence intensity exactly as had been done with the aIL2r labeled cells. Among the so-called positive sort of the McAb unlabeled GAM-FITC labeled control, only 1% of progenitor cells were collected.

Effects of IL2 and aIL2r McAb on colony formation. The effects of r-IL2 on myeloid and erythroid colony formation by CML cells (three cases) and normal bone marrow (four cases) were examined. The addition of rIL2 (500 U/mL) did not alter the myeloid colony forming efficiency in cultures with or without colony stimulating factor, nor did it modify the plating efficiency of BFU-e (Fig 2). Moreover, IL2 alone was inactive in stimulating colony growth.

Blocking of the receptors with 2.5 μg/mL aIL2r McAb exerted no effect on CML myeloid colony growth (CFU-GM), whereas BFUe growth was slightly reduced. This inhibition of BFUe formation (due to aIL2r McAb presence in culture) could not be overcome by the concomitant addition of high concentrations of r-IL2 (500 U/mL) to the cultures (Table 4).

Table 2. Differential Count of aIL2 Receptor Positive and Negative Sorted CML Cell Fractions

Fraction	Unfractionated	aIL2r Positive
Blast cell	53.5	65.6
Promyelocyte	3.1	1.2
Myelocyte	0	0
Metamyelocyte	0	0
Neutrophil	0.2	0.4
Basophil	0	0
Eosinophil	0	0
Monocyte	25.8	20.8
Lymphocyte	2.0	0.8
Unclassified	15.3	11.2
Sudan black	ND	78%
Alpha-naphthyl-esterase	ND	0
E rosette forming	0	0

CML cells were incubated for 18 hours to induce IL2 receptor expression and then subjected to fluorescence activated cell sorting. The differential analysis was made on a total number of 500 cells. The results are expressed as percentages. ND, not done.

Binding of radiolabeled ^{125}I-IL2. To assess the binding properties of the IL2 receptors expressed by CML cells, we performed affinity studies with radiolabeled ^{125}I-IL2. Serial dilutions up to a concentration of 100 pmol/L radiolabeled IL2, which fully saturates high affinity receptors on PHA stimulated peripheral blood cells (PHA-PBL)[4] did not show any significant binding of IL2 to the induced CML cells. Only in the presence of excessive concentrations of ^{125}I-IL2 (ie, 1,500 pmol/L) was some specific binding measured. These results suggest that the IL2 receptor induced on CML cells were predominantly, if not all, of low affinity.

DISCUSSION

The regulatory role of the polypeptide hormone IL2 in the proliferation of activated T and B lymphocytes has been well established.[1,2] The use of pure radiolabeled IL2 preparations and monoclonal antibodies reactive with the surface membrane receptors for IL2 has led to a detailed insight into the inducibility and the binding characteristics of IL2 receptors on normal and neoplastic lymphoid cell types.[4,23,24] Activated T and B lymphocytes express two types of IL2 membrane receptors: one with a high affinity and another with a low affinity for the ligand.[4]

Recently, IL2 receptors have been detected on cells of nonlymphoid origin as well, ie, a subpopulation of in vitro activated peripheral blood monocytes[5,6] and also on AML cells of diverse FAB types in nearly all patients investigated.[7] This raised the intriguing possibility that IL2 receptors also have a regulatory role in the proliferation of AML cell growth. In the present report we demonstrate that IL2 receptors can be induced on a majority of CML (chronic phase) cells during incubation in suspension.

The induction of the IL2 receptors on the CML cells did

Table 3. Numbers of CFU-GM, BFUe, and CFU-GEMM in IL2 Receptor Negative and Positive Cell Fractions of CML Cells and Normal Marrow

Sorted Fractions	Nucleated Cells ($\times 10^5$)	Nucleated Cells %	CFU-GM	BFUe	CFU-GEMM
Patient No. 1 (BM)					
Unfractionated	1	(100)	481	464	41
aIL2r negative	0.497	(49.7)	10	15	0
aIL2r positive	0.503	(50.3)	769	592	24
Patient No. 2 (BL)					
Unfractionated	1	(100)	704	319	47
aIL2r negative	0.586	(58.6)	139	97	58
aIL2r positive	0.414	(41.4)	747	873	116
Patient No. 3 (BL)					
Unfractionated	1	(100)	236	651	72
aIL2r negative	0.724	(72.4)	113	581	29
aIL2r positive	0.276	(27.6)	143	561	87
Normal bone marrow No. 1					
Unfractionated	1	(100)	256	ND	ND
aIL2r negative	0.891	(89.1)	215	127	ND
aIL2r positive	0.109	(10.9)	4	1	ND
Normal bone marrow No. 2					
Unfractionated	1	(100)	ND	895	ND
aIL2 negative	0.894	(89.4)	228	652	ND
aIL2r positive	0.106	(10.6)	4	35	ND

Cells of three CML patients and two normal subjects were first T cell depleted, then stained with the McAb aIL2r and GAM/FITC and sorted into negative (channels 1 to 90) and positive (channels 91 to 255) fluorescent populations. The fractions were cultured to assess the number of hemopoietic progenitors. Colony forming cells are expressed as absolute numbers.

Abbreviations: BM, bone marrow; PB, peripheral blood; ND, not done.

not require specific activating agents such as the lectin PHA or the phorbol ester TPA. This is a property that CML cells share with certain cases of AML, but which distinguishes them from T and B lymphocytes.[1,2,7] Frequently, IL2 receptor positivity appeared following incubation in medium not supplemented with inducers. Separation of IL2 receptor positive and negative cells and their subsequent incubation in colony assays revealed that the majority of the hemopoietic progenitors (CFU-GM, BFUe, CFU-GEMM) express IL2 receptors after induction (Table 3). Morphological analysis showed that the aIL2r positive fractions mainly contained immature cells (blasts) and monocytes.

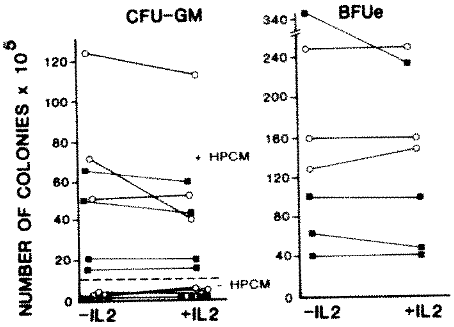

Fig 2. Effect of IL2 on CML and normal marrow colony formation in vitro (CFU-GM, BFUe). + IL2, 500 U/mL IL2 added to the cultures; −IL2, no IL2 added; + HPCM, CFU-GM grown in an assay supplemented with 30% HPCM as the source of colony stimulating factor; −HPCM, no HPCM added. ○, CML (three cases); ■, normal bone marrow (four cases). On the Y axis the number of colonies per 10^5 cells (unfractionated) are plotted.

Table 4. Effect of IL2 Receptor Blocking on CML Colony Growth

	Numbers of CFU-GM (per 10^5 Cells; %) Case 1	Case 2	Case 3
Cultures without addition*	52 (100%)	125 (100%)	71 (100%)
Cultures with aIL2r McAb† (receptor blocking)	133%	89%	70%
Cultures with T6 McAb‡ (no receptor blocking)	107%	88%	65%
	Numbers of BFUe (per 10^5 Cells; %)		
Cultures without addition*	160 (100%)	151 (100%)	285 (100%)
Cultures with aIL2r McAb† (receptor blocking)	37%	27%	30%
Cultures with T6 McAb‡ (no receptor blocking)	70%	74%	81%

The addition of recombinant IL2 (in doses ranging from 50 to 500 U/mL) concomitantly with the aIL2r produced no effect.

*Colonies in nonsupplemented cultures are expressed as mean numbers per 10^5 cells (triplicate cultures) and set at 100%.

†Anti IL2 receptor McAb (2.5 μg/mL) was added to the cultures and colony numbers expressed as percentage of control values (without addition).

‡Control McAb of the same Ig subclass (T6, IgG1) (2.5 μg/mL) was added to the cultures and colony numbers expressed as percentage of data from cultures without addition.

It is noteworthy that the sum of the recoveries of the colony-forming cells from the IL2 receptor positive and negative fractions exceeded the total numbers of the unfractioned cell samples. This was true for CFU-GM and BFUe in all CML experiments and for CFU-GEMM in two of three experiments. The reason for the higher colony forming cell yields following cell separation has not been investigated, but could relate to the removal of inhibiting accessory cells from the IL2 receptor positive cell fraction. Support for this explanation comes, for instance, from cell separation studies on CFU-GEMM, BFUe, and CFU-GM, in which the removal of T cells and mature myeloid cells resulted in increased cloning efficiencies and elevated recoveries of hemopoietic progenitors.[25,26] The presence of IL2 receptors on a large portion of CML cells and particularly CML progenitors suggests a possible role for these receptors in cell proliferation. Normal bone marrow cells and progenitors completely lacked the capacity to express IL2 receptors under identical experimental conditions. The absence of IL2 receptors on normal hemopoietic precursor cells, on one hand, and their expression on human AML and CML cells, on the other, would then suggest this phenotypic trait to be specifically related to the transformed status of the myeloid cells.

Colony cultures for myeloid and erythroid precursors did not provide evidence for a function of IL2 as a growth factor in CML. The colony-forming efficiency of CFU-GM and BFUe in CML was essentially unchanged upon addition of IL2 under various conditions. Blocking the receptors with a IL2r McAb at a concentration that does suppress T cell, and B cell[24] colony growth did not exert any inhibitory effect on CML myeloid colony formation. Although a slight reduction of BFUe growth was observed in the presence of the blocking aIL2r McAb, this suppression was not reversed by adding high concentrations of IL2 (competing with the McAb for receptor binding) concomitantly to the cultures. Thus these studies do not provide positive indications for a functional role of IL2 receptors in CML cell proliferation. The fact that colony growth was not modified following exposure of the cells to rIL2 or to aIL2r could be in good agreement with the findings that virtually no high affinity receptors for IL2 were detectable on the CML cells. The question why IL2 receptors are typically inducible on AML cells and on CML hemopoietic progenitors but not on normal bone marrow (precursor) cells is intriguing. For the time being, their potential pathophysiological role remains uncertain and requires further investigation. In future experiments the question should be addressed as to whether the IL2 receptors on CML cells are structurally of low affinity and whether certain in vitro conditions (eg, prestimulation with special growth factors) will permit their conversion to high affinity receptors. In the latter case, it will be of interest to further investigate the regulatory role of IL2 on the proliferation of CML clonogenic cells, in association with that of other stimuli.

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The Isolation of Human Platelet Factor V

By Robert W. Viskup, Paula B. Tracy, and Kenneth G. Mann

Human platelet factor V has been isolated using either a monoclonal or polyclonal antibody directed against human plasma factor V. The largest peptide observed upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified human platelet factor V comigrates with purified human plasma factor V. However, a significant portion of the isolated protein is represented by peptides of lower apparent molecular weight (M_r). These lower M_r species that copurify with platelet factor V have been shown to be platelet factor V components by their immunological cross-reactivity with monoclonal and polyclonal antibodies to purified human plasma factor V. Platelets isolated from whole blood drawn directly into inhibitors to prevent proteolysis and platelet activation demonstrate the pattern of fragmented platelet factor V. The components of purified platelet factor V demonstrate apparent M_r ranging between 115 K and 330 K and are detectably different from the intermediates and end products observed during the thrombin cleavage of single-chain plasma factor V. Upon treatment with thrombin the platelet factor V components are cleaved and the end products are indistinguishable from those obtained upon thrombin activation of plasma factor V to plasma factor Va. Examination of the components by immunoblotting demonstrates that some of the cleavages which have occurred in the platelet factor V molecule are within the 150-K activation peptide. Bioassay indicates that platelet factor V exists as a procofactor and cleavage by thrombin yields the active cofactor, platelet factor Va. These data suggest that human platelet factor V is stored in the platelet as a partially fragmented procofactor that can be activated by thrombin to yield human platelet factor Va, the active cofactor in the human prothrombinase complex.

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FACTOR V is a single-chain high–molecular weight (M_r) plasma protein that is cleaved by thrombin to yield factor Va, the activated form of factor V. Factor V/Va has been shown to play an essential role in blood coagulation by its involvement in the prothrombinase complex that consists of factor Va, factor Xa, an appropriate membrane surface, and divalent metal ions.[1-4] Although factor Xa alone can catalyze the cleavage of prothrombin to thrombin in solution, its incorporation into the prothrombinase complex yields an increase of 278,000-fold in the rate of thrombin generation.[5] In vitro, phospholipid vesicles have been utilized as the surface which accelerates the conversion of prothrombin to thrombin catalyzed by prothrombinase,[5-7] whereas in vivo the platelet membrane is one surface where assembly likely takes place.[8-12]

Plasma factor V/Va has been shown to be produced by perfused rat liver.[13-15] Recently, our laboratory has demonstrated the de novo synthesis of factor V/Va in bovine aortic endothelium.[16] Chiu et al[17] have reported its production in guinea pig megakaryocytes and Wilson et al[18] have shown it to be synthesized in a human hepatocarcinoma cell line, Hep G2. The presence of factor V antigen in human megakaryocytes,[19] platelets,[19,20] monocytes,[21] and lymphocytes[21] has been reported. Approximately 18% to 25% of the human factor V/Va found in whole blood is contained within the platelet.[20] Platelet factor V is released from the platelet upon activation by thrombin, although the amount of platelet factor V/Va released is not sufficient to saturate the platelet with respect to its prothrombinase activity.[12]

Morrison has reported that plasma factor Va may not be used in the platelet-dependent activation of prothrombin in plasma.[22] Recent work by this laboratory[23] indicates that the platelet provides an important pool of factor V/Va for in vivo prothrombinase complex activity. We have identified a family with an inherited bleeding disorder, Factor V Quebec. The only functional abnormality evident was inactive platelet factor V. Even though prothrombinase complex assembly and activity was completely normal in the presence of added purified clotting factors, this family exhibited a severe bleeding disorder. This not only points out the importance of platelet factor V in the hemostatic process but also raises the possibility that platelet factor V may be a different gene transcription product from plasma factor V. Another observation that demonstrates the importance of platelet factor V in hemostasis has been reported by Nesheim et al.[24] In this instance a plasma factor V inhibitor reduced plasma factor V activity to <1% of normal. Despite this apparent compromise of the hemostatic system, the subject did not bleed abnormally following surgical challenge. Normal platelets isolated from whole blood to which the purified antihuman factor V antibody had been added demonstrated no effect from exposure to the inhibitory antibody. These platelets, upon thrombin stimulation, supported normal prothrombinase activity in the presence of exogenously added factor Xa and the inhibitor. This implies that the platelet factor V was protected from the inhibitory antibody and again implicates platelet factor V as playing a very important role in hemostasis. This recent evidence points to the possibility that even though platelet factor V is not the major pool of factor V present in the circulation, it may be the important species in vivo. We have undertaken this study to isolate and examine human platelet factor V.

MATERIALS AND METHODS

Reagents. Prostaglandin E_1 (PGE_1), ε-amino-*n*-caproic acid (ε-ACA), soybean trypsin inhibitor (STI), phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), *N*-ethylmaleimide (NEM), leupeptin, Protein A, thromboplastin, Triton X-

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100, and bovine albumin (essentially fatty acid free) (BSA) were purchased from Sigma, St Louis. Benzamidine HCl was obtained from Aldrich, Milwaukee; polyethylene glycol (PEG) 8,000 was from J.T. Baker Chemical, Phillipsburg, NJ; Iodogen was from Pierce, Rockford, IL; sodium heparin (from beef lung, 10,000 USP units/mL) was from Organon, W Orange, NJ; rabbit antimouse IgG was from Cappel, Cochranville, PA; and normal mouse sera was from Pel-Freeze, Rogers, AR. QAE-cellulose was purchaed from Schleicher and Schuell, Keene, NH. All other reagents were of the highest analytical grade available.

Proteins. Homogenous, single-chain human factor V was prepared utilizing a hybridoma antibody as previously described by Katzmann et al[25] with modifications as described by Litwiller et al.[26] Factor Va was prepared by the incubation of factor V with catalytic amounts of thrombin.[27] Bovine thrombin was prepared as described by Lundblad et al.[28]

^{125}I-Protein A and ^{125}I-rabbit antimouse IgG were prepared by the Chloramine T method[29] or Iodogen.[30] The radiolabeled proteins were separated from the other iodination products by chromatography on G-25 Sephadex. Mouse ascites were used as the source of murine monoclonal antibody directed against human plasma factor V. This antibody, designated αHFV-7, has been previously characterized by Foster et al[31] and was shown to react with human plasma factor V but not human plasma factor Va. Recent work has shown that the epitope is contained within the activation peptide of M_r 150,000 that has been designated component C1, using the nomenclature suggested by Nesheim et al[32] for bovine factor V.

A polyclonal antibody directed against human plasma factor V/Va was obtained from burro antisera previously described by Tracy et al.[20] IgG was prepared by precipitation with solid ammonium sulfate to 35% saturation followed by chromatography on DEAE-cellulose (10 mmol/L Tris, 40 mmol/L NaCl, pH 8.5). Normal burro IgG was purified in the same manner. These partially purified IgG fractions were subsequently used for immunoblotting. IgG specific for human plasma factor V was purified from the burro polyclonal antibody as described by Nesheim et al[24] and covalently coupled to Sepharose to yield an antihuman plasma factor V immunoabsorbant. Briefly, partially purified burro polyclonal antihuman plasma factor V IgG was applied to a human plasma factor V-Sepharose column and the bound material (specific antihuman factor V IgG) was eluted with 3 mol/L NaSCN. The fractions containing factor V inhibitory activity were pooled, concentrated, and covalently coupled to CH-Sepharose 4B to yield a specific polyclonal antihuman plasma factor V immunoabsorbant to be subsequently utilized for the purification of platelet factor V. A nonimmune Sepharose column was also prepared by coupling normal burro IgG to CH-Sepharose 4B and was used as a control immunoaffinity column.

Factor V bioassay. A two-stage bioassay was used as previously described by Nesheim et al[27] using human factor V–deficient plasma prepared by immunoabsorption of whole plasma on antihuman factor V-Sepharose.[25] Factor V activity was determined by comparing the clot times with those obtained from a standard curve using a normal human plasma pool as the source of factor V. The ratio of the measured factor V activity after and before the intentional activation by thrombin is termed the activation quotient and provides an estimate of the degree to which the factor V sample has been unintentionally activated. Activation quotients of greater than 20 usually indicate a relatively minor amount of unintentionally activated factor V in the sample.

Platelet isolation. Fresh platelet rich plasma (PRP) was obtained from the American Red Cross Blood Center, Burlington, VT. Within approximately 25 minutes after beginning the phlebotomy, an inhibitor cocktail was added to the PRP (1 vol of inhibitor cocktail: 29 vol PRP) such that the final concentration of inhibitors was as follows: 5 mmol/L EDTA, 3.3 mmol/L benzamidine, 1 mmol/L ε-ACA, 20 μg/mL STI, 10 μmol/L PMSF, 2 U/mL heparin, 2.5 μmol/L PGE_1, and 2 mmol/L NEM. Diisopropyl fluorophosphate was subsequently added to a final concentration of 1 mmol/L. All subsequent steps were performed at room temperature using polypropylene tubes and pipettes. The PRP containing inhibitors was centrifuged at 190 × g for 15 minutes to remove red blood cell contamination evident in some of the Red Cross PRP. The PRP was then centrifuged at 1000 × g for 15 minutes to yield platelet poor plasma (PPP) and a platelet pellet. The platelet pellet was gently resuspended using a polypropylene pipette to approximately 40% of the original volume of PRP in Orloff and Michaeli wash buffer (0.127 mol/L NaCl, 4 mmol/L KCl, 30 mmol/L glucose, 10 mmol/L NaH_2PO_4, and 0.35% BSA)[33] containing the inhibitors. The resuspended platelets were washed three times. Platelets were also isolated from blood drawn directly into inhibitors. Approximately 30 mL of blood was drawn using a 19-gauge infusion set directly into a syringe containing anticoagulant (1 vol acid-citrate-dextrose: 6 vol blood) and protease inhibitors as described above. The platelets were then isolated as previously described. Blood was also drawn and allowed to clot to yield normal serum. All blood collection accomplished for this research was reviewed and approved by the Vermont Human Studies Committee.

Isolation of platelet factor V. Washed platelets were resuspended to approximately 4×10^9 platelets/mL in Orloff and Michaeli wash buffer containing inhibitors but not BSA. In addition, leupeptin and DFP were added to final concentrations of 0.5 mmol/L and 1 mmol/L, respectively. All platelet counts were performed using a ZM Coulter Counter (Coulter Electronics, Hialeah, FL).

Platelet lysates were prepared by the addition of Triton X-100 to a final concentration of 1% (vol/vol) followed by sonication. The sonication was repeated three times with one minute of sonication on ice followed by two minutes of cooling. The platelet lysate was then centrifuged at 18,000 × g for 20 minutes at 4°C. To the supernatant, which contained platelet factor V, an equal volume of buffer (25 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 1 mmol/L benzamidine, pH 7.5), an equal volume of cold H_2O, and a small amount of washed QAE-cellulose (~10 mL) were added as previously described for the purification of bovine and human plasma factor V.[25,27] The mixture was stirred in the cold for 30 minutes. The platelet factor V bound to the QAE-cellulose was eluted as previously described.[25,27] The fractions containing platelet factor V activity were pooled and applied to a nonimmune burro IgG Sepharose column (~2 mL bed volume, prepared as described) equilibrated with 0.3 mol/L NaCl, 20 mmol/L imidazole, 5 mmol/L $CaCl_2$, 1 mmol/L benzamidine, pH 6.5). The flowthrough from the nonimmune burro IgG Sepharose column containing the platelet factor V activity was immediately applied to the specific burro polyclonal antihuman factor V immunoabsorbant (~2 mL bed volume, prepared as described) equilibrated in the same buffer as the nonimmune column. The column was then washed until the A_{280} reached baseline. The specific burro polyclonal antihuman factor V immunoabsorbant column was then washed with a minimum of 4 column volumes of 1.0 mol/L NaCl, 20 mmol/L imidazole, 5 mmol/L $CaCl_2$; 1 mmol/L benzamidine, pH 6.5, to remove nonspecifically bound protein. The absorbance of the eluant was monitored. After it reached baseline the bound human platelet factor V was eluted with 4 column volumes of 3 mol/L NaSCN, Tris-Saline, pH 7.4. The eluted human platelet factor V was dialyzed versus 0.2 mol/L acetic acid and lyophilized to dryness. Washed platelets were also lysed by the addition of 10% hot sodium dodecyl sulfate (1 vol SDS:1 vol platelets) or made 50% in acetic acid, lyophilized, and subsequently subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting.

Immunoblotting. After SDS-PAGE on Laemmli 5% to 15% slab gels,[34] immunoblotting was performed as described by Towbin et al.[35] Transfer of the proteins from the polyacrylamide gel onto the nitrocellulose was accomplished using 100 volts for five hours in a Transphor (Hoefer, San Francisco, CA), using cooling coils to keep the temperature of the buffer at approximately 8°C. After transfer, the nitrocellulose sheets were placed into 0.15 mol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4 (Tris-Saline), containing 1% BSA and 0.002% thimerosal to block nonspecific binding. Following transfer, the polyacrylamide gels were stained with Coomassie brilliant blue R to check for the completeness of transfer. Prestained high-M_r standards were used to assist in visualizing the completeness of transfer and to allow calculation of the molecular weight of proteins on the nitrocellulose.

Human plasma and platelet factor V/Va were visualized on the nitrocellulose by incubating the nitrocellulose with a primary antibody (burro polyclonal antihuman plasma factor V or a murine monoclonal directed against human plasma factor V), followed by washing and incubation with either ^{125}I-Protein A (used to visualized burro IgG) or ^{125}I-rabbit antimouse IgG (used to visualize murine monoclonal IgG). Control immunoblots were performed using nonimmune burro IgG or normal mouse serum followed by the appropriately radiolabeled protein. The unbound radiolabel was then washed off and the dried nitrocellulose subjected to autoradiography at −70°C using Kodak (Rochester, NY) XAR-5 film with DuPont (Wilmington, DE) Cronex Lightning Plus intensifying screens. All primary antibodies and radiolabeled proteins were added in Tris-Saline, pH 7.4, containing 1% BSA and 0.002% thimerosal for three hours at room temperature. The washing consisted of one 10-minute wash with Tris-Saline, pH 7.4; one 20-minute wash with Tris-Saline, pH 7.4, containing 0.05% NP-40; and a 10-minute wash with Tris-Saline, pH 7.4.

RESULTS

Chromatographic identity of human platelet and plasma factor V. It has been shown previously using radioimmunoassay that the burro antihuman factor V antibody recognizes both human plasma and platelet factor V/Va and exhibits no cross-reactivity with other plasma proteins.[20] Therefore, for the purification of human platelet factor V, conditions similar to those used for the purification of human plasma factor V were employed. Platelet lysate containing platelet factor V and PPP containing plasma factor V were isolated from PRP obtained from the American Red Cross to which inhibitors had been added as described. The plasma from the PRP was treated with $BaCl_2$ and PEG 8,000 as previously described for the purification of bovine and human plasma factor V.[25,27] Both the plasma and platelet lysate were individually batch-absorbed to QAE-cellulose and subsequently eluted by the stepwise addition of 20 mmol/L imidazole, 5 mmol/L $CaCl_2$, 1 mmol/L benzamidine, pH 6.5, containing (*a*) no NaCl, (*b*) 0.1 mol/L NaCl, and (*c*) 0.3 mol/L NaCl.[25,27] The eluates were assayed for factor V activity and both the platelet and plasma factor V activity were eluted in step (*c*) above. The fractions containing either platelet or plasma factor V activity were concentrated by precipitation to 70% saturation with $(NH_4)_2SO_4$ and redissolved in Tris-Saline, 5 mmol/L $CaCl_2$, pH 7.4. Both were diluted so the activity per milliliter was identical (6 units/mL). Approximately 12 units of platelet or plasma factor V activity were applied to a 1-ml bed-volume murine monoclonal antihuman factor V immunoabsorbent column routinely used for plasma factor V purification.[25] The platelet or plasma factor V activity was retained by the column. The column was then washed and the bound plasma or platelet factor V activity was then eluted with 1.75 mol/L NaCl, 20 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, pH 7.4. Similar recoveries of the pooled peaks of plasma factor V activity (47%) and of the platelet factor V activity (49%) were observed as determined by factor V bioassay. When the 0.3-mol/L NaCl eluates from the QAE-cellulose chromatography step containing either platelet or plasma factor V activity were applied to a nonimmune burro IgG Sepharose column used as a control, the factor V activity was not retained and was recovered in the column flowthrough. The application of partially purified platelet or plasma factor V to the specific burro antihuman factor V Sepharose column also resulted in the binding of both factor V species. Since elution from the polyclonal antibody column was accomplished with 3 mol/L NaSCN and Tris-Saline, pH 7.4, the bioactivity of the eluates from the specific burro antihuman factor V Sepharose column could not be determined.

Purified human platelet factor V. Preliminary studies of human platelet factor V in platelet lysates demonstrated multiple bands when immunoblotted with the burro antihuman factor V antibody. Immunoaffinity chromatography using a monoclonal antibody directed to a single epitope would selectively yield single-chain factor V and the limited number of fragments bearing the relevant epitope. The specific burro antihuman factor V antibody coupled to Sepharose was used to isolate platelet factor V in order to maximize the number of fragments purified. Table 1 shows three typical purifications of human platelet factor V after elution from QAE-cellulose. The activation quotient is approximately 20, indicating that the platelet factor V is activated by thrombin to platelet factor Va. This suggests that platelet factor V is stored as the procofactor and thrombin is able to activate the procofactor to platelet factor Va, the active cofactor in the prothrombinase complex.

Figure 1 is a diagrammatic representation of the postulated intermediates and end products of the thrombin cleavage of human plasma factor V observed in our laboratory.[25] The nomenclature is that used by Nesheim et al[32] for the cleavage pattern observed with bovine plasma factor V. This nomenclature will be utilized in referring to the human plasma factor V components. The apparent M_r are those determined by this laboratory for human factor V/Va.[25] An M_r of 150,000 was assigned to human activation peptide C1 as this species demonstrates an increased M_r on polyacrylamide gels when compared to the bovine fragment C1.

Table 1. Purification of Human Platelet Factor V from QAE-Cellulose

Platelet Preparation	A_{280}	Total Activity* (Units)	Specific Activity (Units/A_{280})	Activation Quotient†
1	29.0	91	3.14	26
2	26.8	84	3.13	21
3	32.2	129	4.01	18

*Activity was measured with factor V-deficient plasma after activation by thrombin.

†Ratio of activity after and before activation. Platelet lysate was batch-absorbed as described and the platelet factor V activity eluted with 0.3 mol/L NaCl, 20 mmol/L imidazole, 5 mmol/L, $CaCl_2$, 1 mmol/L benzamidine, pH 6.5.

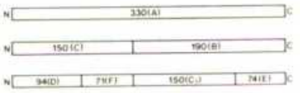

Fig 1. A model of the proposed major intermediates and end products of thrombin-catalyzed activation of human plasma factor V. The nomenclature and sequence alignment are taken from that used previously for bovine plasma factor V,[32] and the molecular weights are those determined by this laboratory for human plasma factor V and its thrombin-catalyzed cleavage products.[25]

Components D and E are assigned the same M_r as their bovine counterparts. Even though the human NH_2 terminal component D demonstrates an M_r of greater than 94,000 on reduced SDS-PAGE, preliminary determinations using analytical ultracentrifugation gives an M_r of approximately 94,000 for human component D (Jenny and Mann, unpublished data).

Figure 2A illustrates an immunoblot of purified human plasma factor V, purified human plasma factor V activated with thrombin, and three different preparations of purified human platelet factor V. The primary antibody was burro antihuman factor V, which recognizes both human plasma factor V, factor Va, and the factor V fragments. The purified human platelet factor V lanes reveal numerous components. The largest molecular weight component comigrates with purified plasma factor V (M_r 330,000). However, the lower-M_r components do not correlate with any of the thrombin or factor Xa activation intermediates[36] or with products of cleaved human plasma factor V/Va. The band at M_r 190,000 is slightly smaller than component B and the two bands migrating with M_r similar to component C1 (155,000 and 140,000) are slightly larger and smaller, respectively, than component C1.

Quantitative immunoblot analysis of the band intensities can be misleading due to the fact that when immunoblotting with a polyclonal antibody, some of the lower-M_r forms of factor V may be recognized more easily than the intact single-chain molecule, making the low-M_r components appear to constitute a disproportionately larger percentage of the total. Also, as ^{125}I-Protein A or ^{125}I-rabbit antimouse IgG was used to visualize the immunoblots, some of the components which exist at the highest concentration would relatively overexpose the film so that the minor bands could be visualized. This would cause a minor component to appear to constitute a larger percentage than it actually does.

Figure 2B represents an immunoblot of the same samples; however, for this analysis the primary antibody was a murine monoclonal antibody against human plasma factor V (αHFV-7) which has been shown to recognize plasma factor V but not plasma factor Va.[31,37] From the immunoblot it is evident that this monoclonal antibody recognizes single-chain plasma factor V (M_r 330,000) and the activation fragment C1 of thrombin-activated plasma factor V. This antibody recognizes a component in purified platelet factor V which comigrates with single-chain plasma factor V (330,000), a band at 190,000, and two smaller bands at 125,000 and 115,000. Note that the two bands of 155,000 and 140,000 evident in blotting with the burro antihuman factor V antibody are not evident, demonstrating that they are not related to component C1. Immunoblotting analysis using monoclonal and polyclonal antibodies to human plasma factor V thus illustrates that factor V purified from platelets is largely fragmented.

Visualization of factor V in platelets isolated directly into inhibitors. There was an interval of approximately 25 minutes between the start of phlebotomy by the American Red Cross and the addition of the inhibitors. The presence of multiple components in the purified platelet factor V preparations indicated the existence of substantial proteolytic cleavage of the single-chain high-M_r platelet factor V molecule even in the presence of numerous inhibitors. In order to assess whether extensive proteolysis of platelet factor V might be occurring prior to the addition of inhibitors, platelets were isolated from blood drawn directly into inhibitors and the extent of proteolysis of their platelet factor V was examined.

Washed platelets from blood drawn directly into inhibitors were lysed with hot detergent (SDS) or glacial acetic acid and subjected to SDS-PAGE followed by immunoblotting. Figure 3 demonstrates that platelet factor V is fragmented, even though the blood is drawn directly into inhibitors and the inhibitors are present throughout the entire washing and lysing procedure. The fragments of platelet factor V seen in the lysed platelets are the same as the fragments seen in the purified preparations of platelet factor V. Increasing the concentration of inhibitors by an order of magnitude had no effect on the fragmentation pattern seen on the immunoblots. Plasma from the same individual shows the major species of plasma factor V being component A, intact factor V at M_r 330,000. Serum was also made at the same time with no

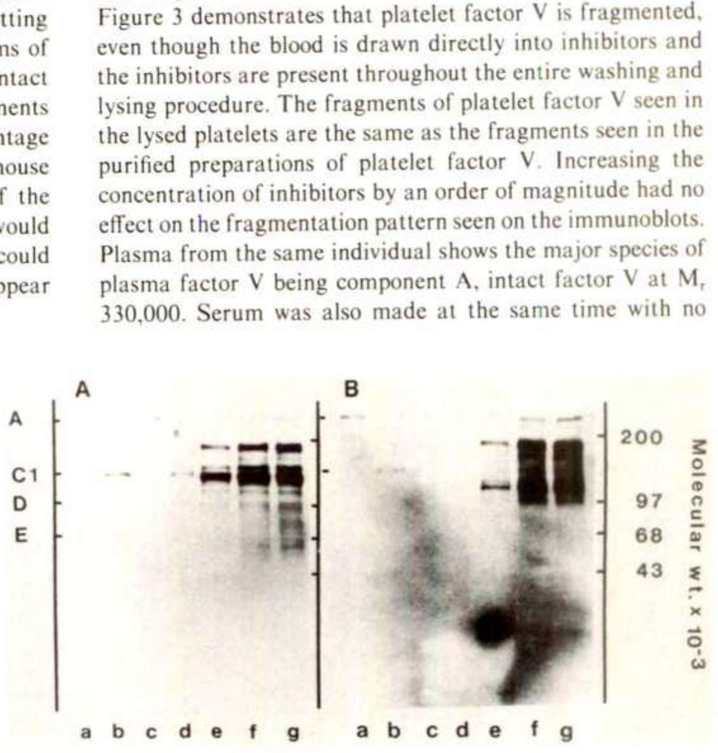

Fig 2. Immunoblot of purified human plasma and human platelet factor V. In (A) the primary antibody was burro antihuman factor V followed by ^{125}I-Protein A. The primary antibody in (B) was a murine monoclonal to human plasma factor V (αHFV-7) followed by ^{125}I-rabbit antimouse IgG. The samples in the various lanes are the same in both panels. Lanes: (a) Purified human plasma factor V, 200 ng; (b) purified human plasma factor V, 200 ng, activated with thrombin to yield human plasma factor Va and the activation peptides; (c) purified human plasma factor V, 100 ng; (d) purified human plasma factor V, 100 ng, activated with thrombin to yield human plasma factor Va and the activation peptides; and (e through g) three different preparations of purified human platelet factor V (~4 μg).

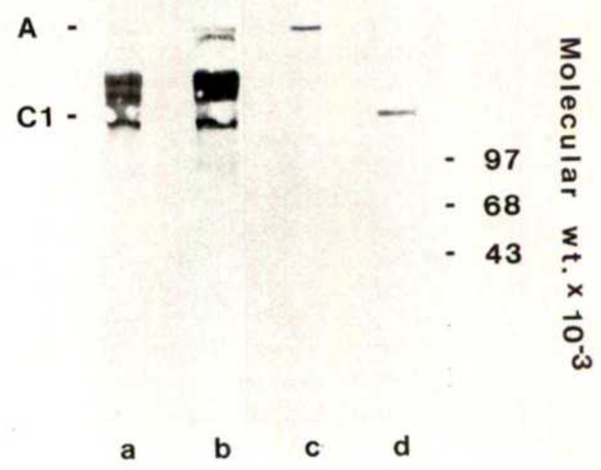

Fig 3. Immunoblot of human platelets drawn directly into protease inhibitors and washed as described. The primary antibody was burro antihuman factor V followed by ^{125}I-Protein A. Lanes: (a) human serum (7.5 μL); (b) human plasma (7.5 μL); (c) washed platelets (5 × 10^7 platelets) lysed with detergent (SDS); and (d) washed platelets (5 × 10^7 platelets) lysed with glacial acetic acid.

inhibitors present and upon immunoblotting demonstrates a band which comigrates with the major immunoblotted band evident in thrombin-activated human factor V (component C1, the large activation peptide, M_r 150,000).

The possibility that the protease inhibitors were not inhibiting the cleavage of platelet-associated factor V during the lysis of the platelets was examined. Human plasma factor V and the various thrombin generated intermediates were mixed and radioiodinated. A mixture of factor V, its high-M_r intermediates, and the factor Va subunits was chosen in order to detect proteolysis of any single component. The ^{125}I-factor V(Va) mixture was incubated with washed platelets (2 × 10^9) that were isolated and washed in the presence of the inhibitor cocktail detailed in Materials and Methods. Following the various incubations detailed below, the ^{125}I-factor V(Va) mixture and lysed platelets were subjected to SDS-PAGE followed by autoradiography. Figure 4 illustrates the results of this experiment. Lane a depicts the ^{125}I-factor V(Va) mixture added to the buffer containing inhibitors followed by the addition of an equal volume of boiling 10% SDS. Lane b depicts the same ^{125}I-factor V(Va) mixture added to washed platelets followed by the addition of an equal volume of boiling 10% SDS. Lane c depicts the lysing of platelets by boiling 10% SDS followed by the addition of the ^{125}I-factor V(Va) mixture. The ^{125}I-factor V(Va) mixture was also added to platelets and the platelets lysed by the addition of Triton X-100 to a final concentration of 1%. After five minutes (lane d), 10 minutes (lane e), and 15 minutes (lane f), the incubation was terminated by the addition of boiling SDS. There is no discernible difference in the ^{125}I-factor V(Va) mixture when added to platelets lysed with either boiling SDS or Triton X-100 followed by boiling SDS. Identical results were obtained when platelets were lysed by the addition of glacial acetic acid (50% vol/vol). These results indicate that the protease inhibitors present are inhibiting any additional cleavage of the factor V which might occur during platelet lysis.

Thrombin activation of isolated platelet factor V. The procofactor nature of the fragments seen in the platelet factor V preparation was examined. Plasma factor V is cleaved by thrombin to yield the active cofactor which functions in the prothrombinase complex, factor Va. The partially purified platelet factor V after the QAE-cellulose chromatography step demonstrated activation quotients[4] of 18 to 26 showing that platelet factor V, the procofactor, was in fact activated to Va, the cofactor, by thrombin. The ability of thrombin to cleave plasma factor V to its active cofactor is retained even when plasma factor V is bound to the monoclonal antibody column routinely used for its isolation.[31] A

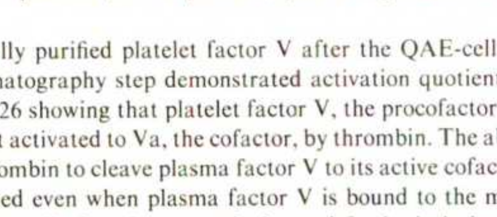

Fig 4. Autoradiography of a ^{125}I-human factor V(Va) mixture incubated with washed platelets. A mixture of ^{125}I-factor V and Va was added to washed platelets (2 × 10^9) which were collected, isolated, and resuspended in protease inhibitors. Following the protocols detailed below for each lane the ^{125}I-factor V(Va) mixture was subjected to SDS-PAGE followed by autoradiography. Lanes: (a) ^{125}I-factor V(Va) mixture plus 10% boiling SDS; (b) ^{125}I-factor V(Va) mixture and platelets followed by the addition of 10% boiling SDS to effect platelet lysis; (c) platelets plus 10% boiling SDS followed by the addition of the ^{125}I-factor V(Va) mixture; (d through f) ^{125}I-factor V(Va) mixture plus platelets which were lysed by the addition of Triton X-100 (1% final concentration) and incubated at room temperature for 5, 10, and 15 minutes respectively, prior to the addition of 10% boiling SDS.

similar approach was used to examine the activation products of platelet factor V. Following the elution of platelet factor V from QAE-cellulose with 0.3 mol/L NaCl, the preparation was then divided into two parts. Both were applied to separate specific burro antihuman factor V Sepharose columns (~1-mL bed volume, prepared as described). One column was washed and the platelet factor V eluted as the procofactor with 3 mol/L NaSCN, Tris-Saline, pH 7.4 as described. The other column was washed with 1.0 mol/L NaCl, 20 mmol/L imidazole, 5 mmol/L $CaCl_2$, 1 mmol/L benzamidine, pH 6.5 and then equilibrated with Tris-Saline, 5 mmol/L $CaCl_2$, pH 7.4. Thrombin was added to the column (3 U/mL), the flow stopped, and the column allowed to remain at room temperature for 30 minutes. The thrombin-cleaved platelet factor V was then eluted with 3 mol/L NaSCN, Tris-Saline, pH 7.4. The fractions were dialyzed v 0.2 mol/L acetic acid, lyophilyzed, and subjected to SDS-PAGE followed by immunoblotting.

Figure 5 is an immunoblot of purified human plasma factor V showing a time course of isolated human plasma factor V activated by thrombin, purified human platelet factor V, and purified human platelet factor V activated by thrombin. The lanes containing the time course of plasma factor V activation by thrombin (lanes b through e) show the disappearance of the single-chain high-M_r factor V molecule (component A, M_r 330,000); the appearance and disappearance of component B, an intermediate; and the appearance of the final end products, component C1 (M_r 150,000), the larger activation peptide, and components D and E which constitute the active cofactor, factor Va. Fragment E is hard to see in the blot because the specific burro polyclonal antihuman factor V antibody does not detect fragment E well. The lane containing purified platelet factor V (lane f) contains a component similar to component B; however, it demonstrates a slightly lower mobility. Although the appearance of component C1 is very evident in the thrombin-activated plasma factor V (lanes b through e), there is no corresponding component evident in the purified platelet factor V (lane f). The two bands migrating in that vicinity in purified platelet factor V (lane f) demonstrate M_r of 155,000 and 140,000 and are slightly larger and slightly smaller than component C1. However, when platelet factor V is treated with thrombin (lane g) a component equivalent to plasma component C1 is evident from both immunological and M_r evaluation. The purified platelet factor V (lane f) demonstrates no bands comigrating with components D and E, the two polypeptides that constitute factor Va, the active cofactor. Thrombin-activated platelet factor V (lane g) contains a platelet component D and a platelet component E. Therefore, even though the purified platelet factor V is partially fragmented, it is still activated by thrombin from the inactive procofactor to the active cofactor, platelet factor Va.

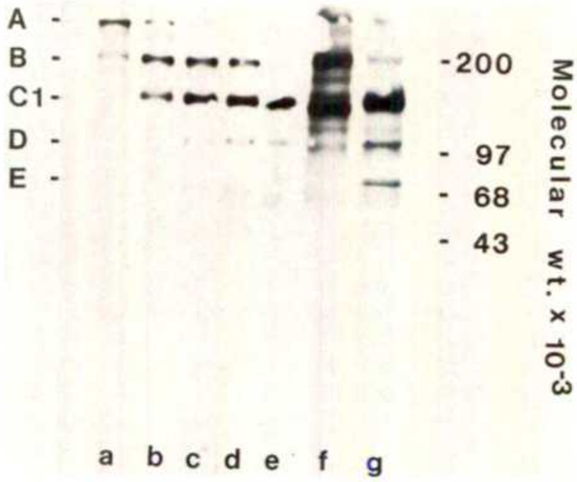

Fig 5. Immunoblot of purified human plasma factor V, purified human plasma factor V incubated with thrombin for various times, purified human platelet factor V, and purified human platelet factor V incubated with thrombin. The primary antibody was burro antihuman factor V followed by ^{125}I-Protein A. Lanes: (a) Purified human plasma factor V (650 ng); (b through e) purified human plasma factor V (650 ng) incubated with thrombin for 10 seconds; 30 seconds, 60 seconds, and 15 minutes, respectively; (f) purified platelet factor V (4 μg); and (g) purified platelet factor V (4 μg) incubated with thrombin as described.

DISCUSSION

Our results demonstrate that purified human platelet factor V can be seen to exist as a single-chain high-M_r entity that comigrates with purified human plasma factor V. Also evident are numerous components identified as arising from platelet factor V by their antigenic cross-reactivity with both polyclonal and monoclonal antibodies to human plasma factor V. Previous work by this laboratory has demonstrated immunological similarity between platelet and plasma factor V using radioimmunoassay[20] and immunofluorescent staining of human platelets and megakaryocytes.[19] Both the single-chain high-M_r human platelet factor V molecule and the lower-M_r components are recognized by thrombin and are cleaved to yield products which appear to be identical to those derived from human plasma factor V after cleavage by thrombin.

Partially purified human platelet factor V when treated with thrombin demonstrates an increased activity in the prothrombinase complex as seen by activation quotients of 18 to 26. This is equivalent to what we find for partially purified preparations of human plasma factor V, indicating that human platelet factor V exists as a procofactor. Our laboratory has observed that factor V synthesized by cultured bovine aortic endothelial cells is largely fragmented.[16] The bovine aortic endothelium factor V also exists as the procofactor, even though partially fragmented, and treatment with thrombin yields the active cofactor. Upon treatment with thrombin, the endothelial cell factor V fragments give rise to end products which appear identical to those observed upon thrombin activation of purified single-chain plasma factor V. Chiu et al[17] have recently reported the isolation of guinea pig megakaryocyte factor V. Their results indicate that guinea pig megakaryocyte factor V exists as a single-chain high molecular weight entity. We have not been able to obtain single-chain platelet factor V as a major fraction of factor V from platelets isolated from blood collected directly into inhibitors and immediately lysed with either detergent (SDS) or glacial acetic acid. These data indicate that the platelet factor V appears to be stored in platelets as a partially fragmented procofactor that can be activated by thrombin to the active cofactor, platelet factor Va. Alternatively, the platelet factor V may be partially cleaved by intracellular platelet proteases while the platelets are being collected and washed, even though protease inhibitors are present throughout the collection and wash. The inhibitors were present external to the platelet membrane during the wash, so if there is any proteolysis of platelet factor V by intracellular proteases we are unable to prevent its occurrence until after the platelets are lysed. Even platelets lysed with SDS or glacial acetic acid show that some of their platelet factor V exists as fragments. The inhibitors utilized were chosen based upon our previous experience in isolating both human and bovine plasma factor V and the recently published isolation of guinea pig megakaryocyte factor V.[17] Ethylenediamine tetraacetic acid (EDTA), NEM, and leupeptin were added to inhibit the calcium-activated neutral protease found in platelets.[38-40]

Examination of platelet factor V with an activation peptide (component C1)-specific monoclonal antibody reveals fragments of M_r >150,000 in the preparation of purified platelet factor V. Also evident are components smaller than component C1 with M_r of 125,000 and 115,000. This observation indicates that some of the cleavages of platelet factor V are occurring within the activation peptide region. This observation agrees with that of Kane et al[41] that lysed human platelets contain a protease which cleaves exogenously added human plasma factor V. They determined that some of the cleavages were occurring in the activation peptide region (fragment C1) and concluded that the activation peptide region of plasma factor V might be susceptible to proteolytic cleavage. These data correlate with the ability of partially purified platelet factor V to be activated by thrombin even though the platelet factor V has been partially cleaved.

Osterud et al[42] have previously suggested that platelets contain an enzyme which will activate platelet factor V. They also proposed that the factor V released from platelets already exists as the active cofactor, factor Va. Osterud et al[42] and Vicic et al[43] have reported that the factor V activity released after platelet lysis was not increased when incubated with thrombin, indicating that the platelet factor V existed as the cofactor, platelet factor Va. These investigators isolated platelets in the absence of protease inhibitors, and after lysing their platelets by either freeze-thawing or by the addition of Triton X-100, they saw only a slight increase in the bioassayable factor V activity after the addition of thrombin. In contrast to those data and in agreement with ours, Kane et al[41,44] and Chesney et al[45] have found that most of the platelet factor V is stored as the procofactor and the addition of thrombin causes an increase in the released platelet factor V activity. These investigators[41] have reported that the soluble fraction of platelets contains a protease that can partially activate purified factor V. They have shown that the platelet factor V released from platelets is released as the procofactor. This points out the importance of inhibiting the endogenous proteases that may act upon platelet factor V when factor V is released from the platelets or the platelets are lysed.

We have taken care to attempt to isolate and lyse our platelets in the presence of a substantial number of various protease inhibitors. Even so, our purified platelet factor V is seen to exist partially fragmented. When platelets are collected directly into inhibitors, washed, and then lysed with SDS or glacial acetic acid keeping inhibitors present throughout, the platelet factor V is still partially fragmented. Many of the cleavage products observed by Kane et al[41] appeared to be the result of peptide bond cleavage in the component C1 region of the factor V molecule. The possibility thus exists that the protease they observed may be responsible for the partial fragmentation we see in platelet factor V.

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Further Studies of the Secretory Pathway in Thrombin-Stimulated Human Platelets

By James G. White and Marcy Krumwiede

The pathway followed by secretory products stored in platelet alpha granules during the release reaction remains controversial. Tannic acid has been used in the present study as an electron-dense stain to follow the secretory process in thrombin-stimulated platelets. Preliminary experiments demonstrated that tannic acid precipitates fibrinogen, and binds osmium tetroxide to fibrinogen and fibrin strands. Examination of platelets fixed at short intervals after exposure to thrombin and incubated in solutions containing tannic acid revealed electron-dense deposits of osmium not apparent in resting platelets. Granules and lumina of channels making up the open canalicular system (OCS) were unstained in discoid cells. However, exposure to thrombin at concentrations of 1 to 5 U/mL for thirty seconds or more resulted in intense staining of alpha granules by osmium. Some granules communicated directly with dilated channels of the OCS, and several were frequently connected to the same canaliculus. The electron-dense substance in swollen granules and channels appeared to be in the process of extrusion through narrow or dilated openings of the OCS onto the platelet surface. Granule-to-granule fusion and formation of sealed vacuoles of fused granule products unstained by tannic acid–osmium were not observed. The findings support the concept that secretion by stimulated human platelets results from development of direct communications between granules and channels of the OCS and subsequent extrusion of products through channel pores to the surrounding medium.

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THE PROCESS OF PLATELET secretion has been a subject of interest since the release reaction was first described.[1-4] Early studies identified a system of tortuous, interconnected canaliculi in resting platelets[5,6] that appeared to serve as conduits for the discharge of storage granule products during secretion.[7,8] A simlar conclusion regarding the role of the open canalicular system (OCS) was reached in a study that demonstrated fibrin strands in the channel system of platelets activated by thrombin in the presence of EDTA.[9,10]

More recent investigations, however, have questioned the process of platelet secretion proposed in the early studies.[11-13] Ginsberg et al used immunofluorescence with monoclonal antibodies to granule products and electron microscopy to follow the secretory process in thrombin-stimulated human platelets.[12,13] Results obtained in their studies suggested that centrally concentrated granules fused together to form closed sacs that migrated to platelet surfaces, attached to the cell wall, and discharged contents to the outside. The findings appeared to confirm other work emphasizing the development of vacuoles in thrombin-stimulated platelets.[14-16] Other agents, such as phorbol myristate acetate[17] and the synthetic diglyceride, oleoylacetyl glycerol,[18] which cause secretion without platelet internal transformation, also stimulate vacuole formation in platelet cytoplasm.

Investigations of bovine platelets have also weakened arguments favoring a central role for the OCS in platelet secretion. Bovine platelets have been shown to secrete the same granule-based products as human cells.[19] However, Zucker-Franklin et al have shown that cattle platelets are virtually devoid of an open canalicular system.[20] Because of the basic similarity in the secretory response of human and bovine cells, they questioned the role of the OCS in the release reaction of either cell type.

The present study has reexamined the hypothesis that the OCS serves as the secretory pathway during the release reaction of human platelets. Tannic acid, often used as an electron dense stain,[21] was found to precipitate fibrinogen. Acting as a mordant, tannic acid selectively deposited osmic acid on fibrin and possibly other products involved in the process of extrusion from granules to channels and from the OCS through pores in the surface to the exterior of platelets stimulated by thrombin.

MATERIALS AND METHODS

General

Blood for this study was obtained from well-characterized normal donors after informed consent. Samples obtained by venipuncture were mixed immediately with citrate–citric acid–dextrose, pH 6.5 (93.0 mmol/L sodium citrate, 7.0 mmol/L citric acid, and 140 mmol/L dextrose) in a ratio of 9 parts blood to 1 part anticoagulant.[22,23] Platelet-rich plasma (C-PRP) was separated from whole blood by centrifugation at 100 g for 20 minutes at room temperature.

Preparation of Washed Platelets

Samples of C-PRP were combined with an equal volume of the citrate anticoagulant and centrifuged at 400 g for 15 minutes at 20°C to pellets.[24] Supernatant was discarded and replaced with Hank's balanced salt solution (HBSS) containing either 0.1 mol/L EDTA or adenosine (5 mmol/L) and theophylline (3 mmol/L). After 30 minutes at 37°C, the pellets were resuspended in the wash solution. The procedure was repeated twice and the platelets resuspended in HBSS with or without 0.1 mol/L EDTA at a final concentration of 5 × 10^5/mL. The washed suspensions were maintained in the 37°C water bath until used in specific experiments.

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Reagents

Bovine thrombin and purified human fibrinogen were obtained from Sigma (St Louis). Reagent grade tannic acid was purchased from Mallinckrodt Chemical Company (St Louis). The reagents were dissolved in cacodylate buffer adjusted to a pH of 7.2.

Experimental Studies

Thrombin and PRP. Samples of C-PRP were fixed for study in the electron microscope before and after exposure to thrombin according to methods described previously.[25,26] Fixation was accomplished by combing the sample at the selected time with an equal volume of 0.1% glutaraldehyde in White's saline (a 10% solution of a 1:1 mixture of [a] 2.4 mmol/L NaCl, 0.1 mmol/L KCl, 46 mmol/L $MgSo_4$, 64 mmol/L $Ca(No_3)_2$, $4H_2O$ and [b] 0.13 mol/L $NaHCO_3 \cdot$ 8.4 mmol/L NaH_2PO_4 and 0.1 g/L of phenol red, pH 7.4).[27,28] After 15 minutes the samples were centrifuged to pellets and the supernatant fixative was removed and replaced with 3% glutaraldehyde in the same buffer. The samples resuspended in the second aldehyde fixative were maintained at 4°C for 30 minutes, then sedimented to pellets. Supernatant was removed and replaced with either 1% osmic acid in Zetterquist's buffer or 1% osmic acid in distilled water containing 1.5% potassium ferrocyanide for one hour at 4°C.[29]

Tannic acid has been used in our previous studies as an electron-dense stain for the open canalicular system of human platelets.[30,31] In the present study the method modified from the procedure reported by Somlyo[32] was used to stain the secretory pathway of human platelets. C-PRP and cells washed and resuspended as described above and exposed to thrombin in the presence or absence of EDTA were fixed initially with 0.2% glutaraldehyde in White's saline followed by sedimentation to a pellet, removal of the supernatant, and replacement with 3% glutaraldehyde in the same buffer for two hours at 4°C. After completion of the initial fixation steps, pellets were washed three times and stored overnight in 0.1 mol/L cacodylate buffer, pH 7.2. The buffer was decanted the following day and replaced with 2% tannic acid in 0.1 mol/L cacodylate buffer, pH 7.2 for four hours at room temperature. The supernatant was decanted, the pellets washed in cacodylate buffer and the samples postfixed in 2% osmic acid alone or combined with 1.5% potassium ferrocyanide for two hours at 4°C.

The system used in previous studies into effects of thrombin on PRP was employed in the present investigation.[25,33] Thrombin at a final concentration of 0.4 or 1.0 U/mL was combined with 1 mL of C-PRP inverted once and left in the 37°C water bath without further stirring. At intervals of 15, 30, and 45 seconds and one, three, and five minutes, samples were fixed as described above.

Thrombin and washed platelets. Suspensions of washed platelets in the presence or absence of EDTA were combined with thrombin, inverted once, and allowed to stand at 37°C without further stirring until fixed at the selected interval. Thrombin was added to 1 mL of suspension for 15, 30, and 45 seconds, and one, three, and five minutes at final concentrations of 1, 3, and 5 U/mL. Fixation was accomplished in the presence or absence of tannic acid as described above.

Tannic acid and fibrinogen. Preliminary studies into effects of thrombin on platelets in C-PRP had suggested that tannic acid acted selectively on fibrinogen and fibrin, resulting in the subsequent deposition of osmium as a dense stain. To examine this possibility, samples of platelet-poor plasma (PPP) and solutions of purified fibrinogen (250 μg/mL) were combined with equal volumes of 2% tannic acid in cacodylate buffer, pH 7.2. Precipitates that formed in both systems were fixed for study in the electron microscope. Other samples of PRP and PPP as well as solutions of fibrinogen were treated with thrombin (1 U/mL) and subsequently fixed in the presence of tannic acid as described above.

Final Preparation for Electron Microscopy

All samples were dehydrated in a graded series of alcohol and embedded in Epon 812. Thin sections cut from the plastic blocks on an ultramicrotome were examined unstained or after staining with uranyl acetate and lead citrate to enhance contrast. Examination was carried out in a Philips (Mahwah, NJ) 301 electron microscope.

RESULTS

Unstimulated Platelets Stained with Tannic Acid

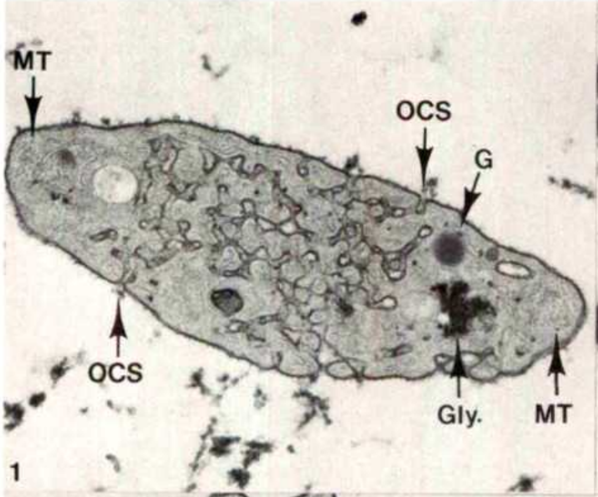
The morphology of well-preserved, resting human platelets has been described in detail in previous publications.[26-28] Resting cells have a characteristic lentiform appearance supported by a circumferential microtubule coiled under the cell surface along its greatest circumference (Fig 1). Channels of the OCS are continuous with the cell surface and form a tortuous, interconnecting lattice of canaliculi throughout the cell. Under the conditions used in this study, tannic acid combines with the exterior coat of the platelet membrane and acts as a mordant to preferentially deposit osmic acid as an electron-dense stain. As a result, the surface of the platelet and channels of the OCS are clearly delineated by dense lines. The lumina of the OCS channels are unstained in the resting platelet.

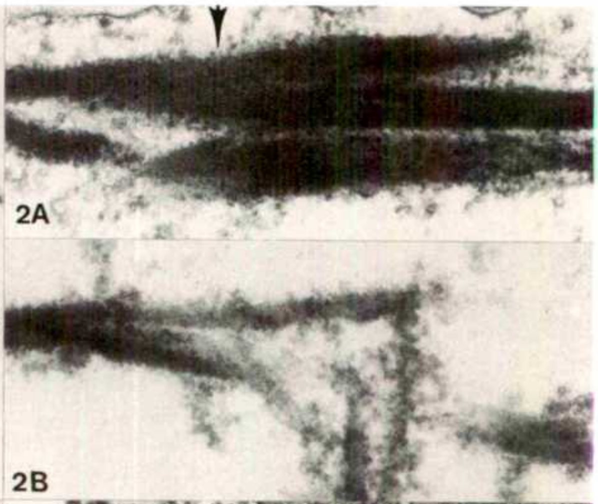
Interaction of Tannic Acid with Fibrinogen and Fibrin

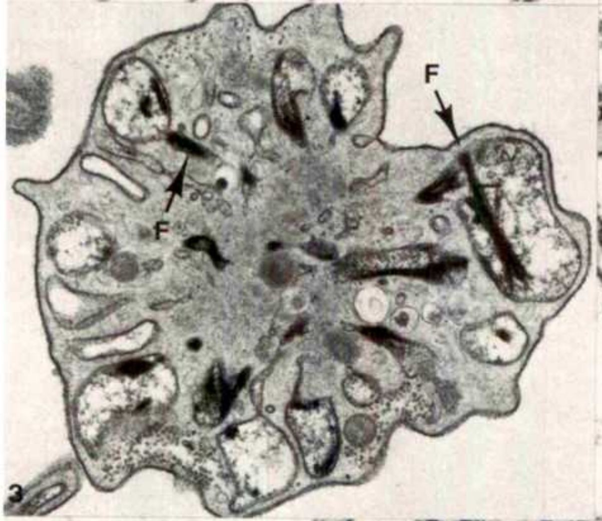
Newly formed and well-developed fibrin strands together with the heavy precipitate around and between platelets in samples of C-PRP exposed to thrombin were intensely stained by osmium (Fig 2A). This finding suggested that tannic acid might selectively, though not exclusively, react with fibrinogen and fibrin. Thin sections of tannic acid–induced precipitates formed in PPP and solutions of fibrinogen revealed intense osmium staining of amorphous and fibrillary material. Fibrin strands formed after addition of thrombin to the fibrinogen solution and subsequently stained with tannic acid during fixation revealed the same selective deposition of osmium on fibrin strands as observed in PRP and PPP (Fig 2B). Platelets from washed preparations exposed to thrombin in the presence or absence of EDTA contained fibers identical in appearance to those formed in PRP, PPP, or fibrinogen solutions in swollen granules and channels of the OCS (Fig 3).

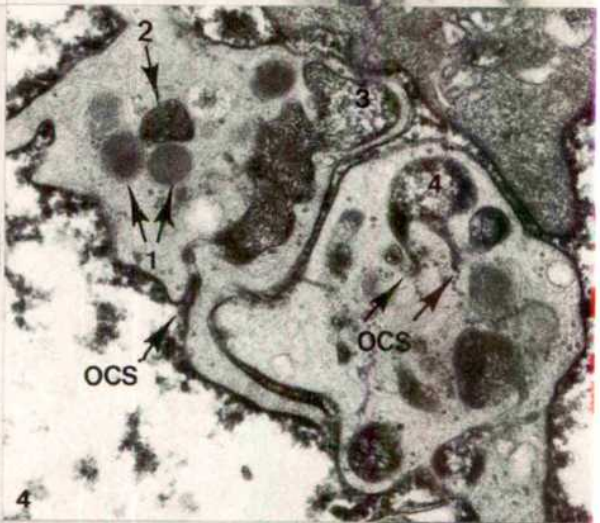
Thrombin-Stimulated Platelets

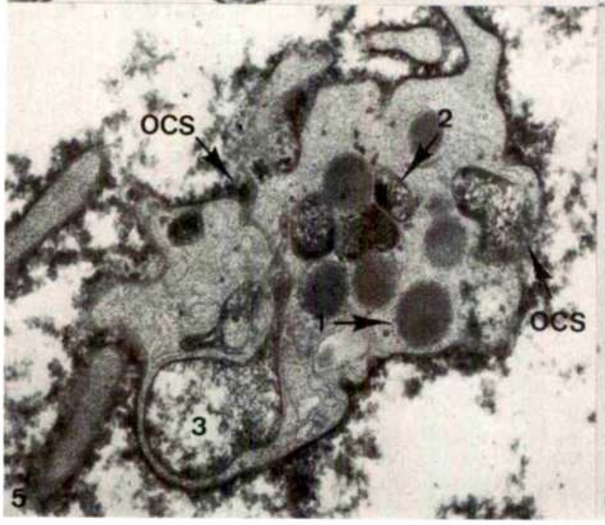
Samples of C-PRP and washed human platelets stimulated by thrombin in the presence of EDTA develop dramatic changes in their morphology. The cells lose their lentiform appearance, become irregular in form, extend numerous pseudopods, and aggregate in the absence of EDTA. At 0.4 U/mL of thrombin, platelet organelles become concentrated in cell centers and enclosed within rings of constricted microtubules. Higher concentrations of thrombin (1 U/mL) cause rapid discharge of granule contents and reduction in their number. As a result, dense spots of actomyosin in which











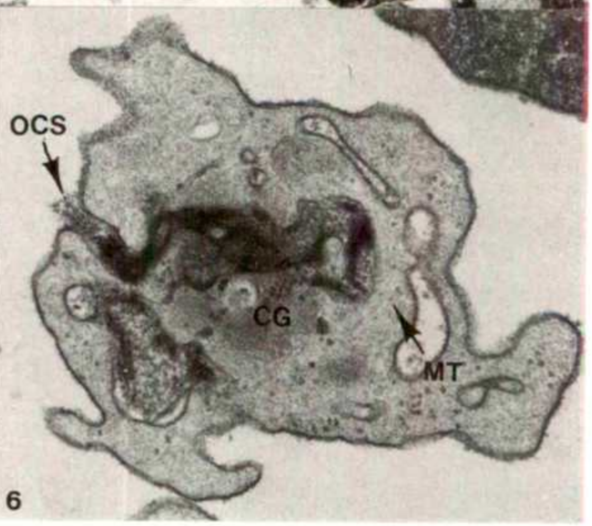


Fig 1. Cross-section of discoid platelet from sample of C-PRP stained with tannic acid (TA) during fixation with glutaraldehyde (glut) followed by osmic acid (OsA). Circular profiles of the microtubule (MT) coils supporting discoid shape are evident at each pole of the cell. Tannic acid has acted as a mordant to deposit osmic acid in the exterior coat covering the exposed surface and channels of the open canalicular system (OCS). The OCS forms a network of interconnected channels stretching from one side of the platelet to the other. Granules (G) and a mass of glycogen (Gly) particles are evident in the cytoplasm. Magnification × 20,000. Fig 2. (A) Fibrin strands in a sample of C-PRP fixed five minutes after incubation with 0.2 U/mL thrombin. Tannic acid has combined with the fibrin strands and with osmic acid, resulting in intense staining of the fibers. Typical periodic cross-striations (↑) are evident (fixation: Glut-TA-OsA). Magnification × 40,000. (B) Fibrin strands formed in sample of purified fibrinogen one minute following exposure to thrombin at 1 U/mL.

centrally concentrated organelles are enclosed in less activated platelets appear more prominent in strongly stimulated cells.

Tannic Acid Staining of Thrombin-Stimulated PRP

Tannic acid–stained platelet aggregates from C-PRP were identical in appearance to unstained control aggregates, except for the presence of osmium black precipitate. Amorphous black material surrounded the aggregates and was deposited between the cells (Figs 4 and 5). Electron-dense material was also present in normal-sized and swollen granules in many platelets. Connections between channels and granules and direct communication between canaliculi and the surrounding plasma were evident. Cytoplasmic structures including mitochondria, granules, glycogen deposits, and microtubule coils were well-preserved in the aggregated cells.

Tannic Acid Staining of EDTA Washed Platelets

Amorphous precipitate was not present outside the thrombin-activated cells from samples of washed platelets resuspended in the presence of EDTA, and aggregates were absent. The platelets, however, revealed the same physical changes observed in thrombin-aggregated cells from PRP (Figs 6 through 12). Many granules were stained intensely by tannic acid–osmium. Other granules were swollen and their content of amorphous stained material appeared diluted (Fig 8). Channels of the OCS were also delineated by electron-dense stain. Some channels were tortuous and narrow and contained little tannic acid. Others were filled by electron-dense material and widely dilated. Communications between granules and OCS channels were evident in many platelets (Figs 6 through 8, 10). The connection appeared to foster swelling of the granule and dilatation of the channel so that recognition of the site of fusion was often obscured. More than one granule was frequently in communication with the same OCS channel (Figs 7 and 8). This relationship frequently resulted in extensive dilatation of the OCS and granules fused to it. Occasionally channel openings onto the surface were dilated, but more often remained constricted as in resting platelets.

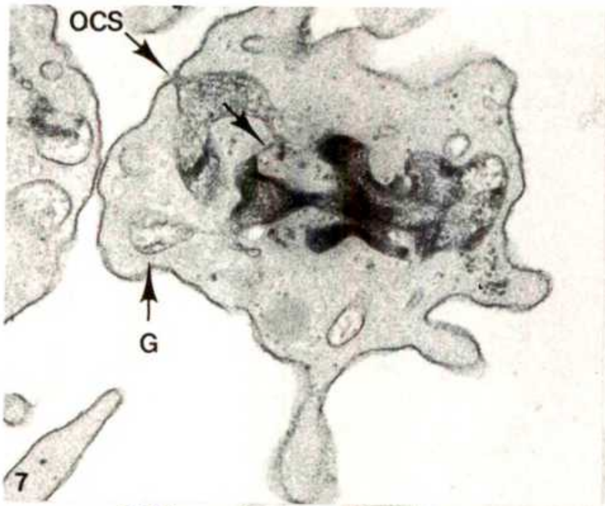
Communications between channels of the OCS and surface membrane in washed platelets exposed to thrombin in the presence of EDTA were identical in appearance to those observed in thrombin-aggregated cells from C-PRP. Some channels were dilated immediately under the cell membrane, while others remained narrow for considerable distances in the cytoplasm. Many were connected to other canaliculi and to granules. Occasionally a single channel opened in more than one place onto the surface membrane of an activated platelet (Figs 10 through 12). The electron-dense material present in channels frequently appeared to extrude into the surrounding medium. Advanced physical changes were most prominent in samples exposed to thrombin at 5 U/mL. The number of affected platelets increased between 15 and 45 seconds after exposure, but all were affected by one minute. Three units/mL of thrombin produced essentially the same results as obtained with 5 units/mL. Fewer platelets revealed the advanced changes seen with 3 and 5 U of thrombin/mL when incubated with thrombin at 1 U/mL. However, there were some, and all platelets revealed shape change and internal transformation at one minute after exposure to thrombin. Each experiment in this study has been repeated four to seven times.

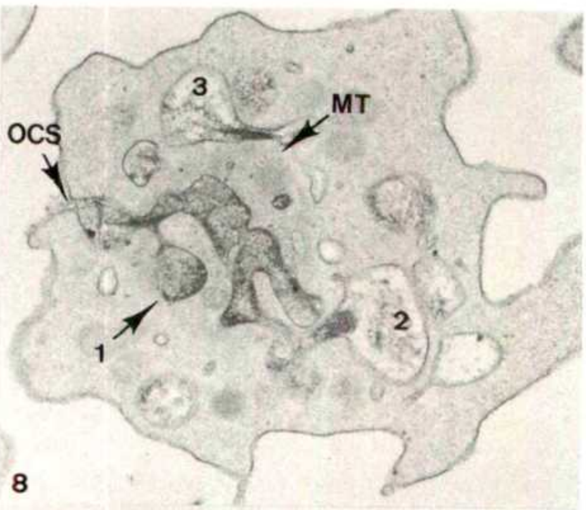
DISCUSSION

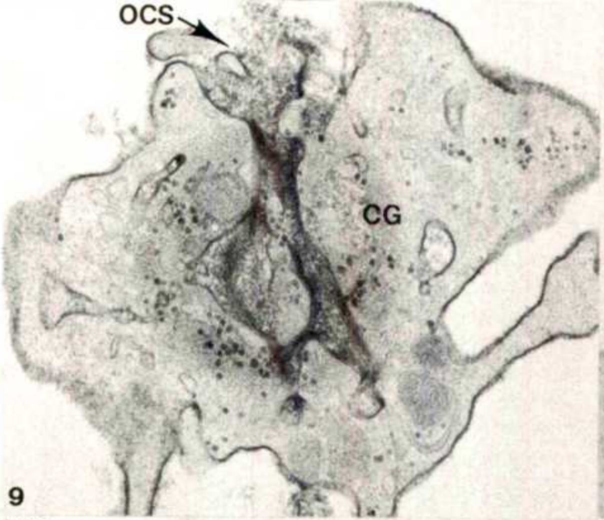
Results of the present study confirm and extend earlier work employing electron-dense tracers, which established existence of the platelet OCS and suggested its role in secretion.[5-9,34,35] In contrast to compounds used previously, tannic acid employed in the present study is not inherently electron-dense.[21,36,37] Instead, it combines with a variety of proteins, glycoproteins, complex carbohydrates, and phospholipids inside and outside of cells, and stabilizes them against extraction during fixation and dehydration.[38-41] Acting as a mordant through gallic acid groups on the molecule, tannic acid selectively deposits osmic acid on chemical or physical substances to which it has attached.[42,43] Cells are ordinarily impermeable to tannic acid, but it may enter the cytoplasm during fixation when detergents are combined with glutaraldehyde.[44] Since its introduction to electron microscopic studies, tannic acid has been used extensively to enhance the contrast of cell membranes and channels connected to the surface, as well as microtubules and microfilaments inside permeabilized cells.[39,40,45,46]

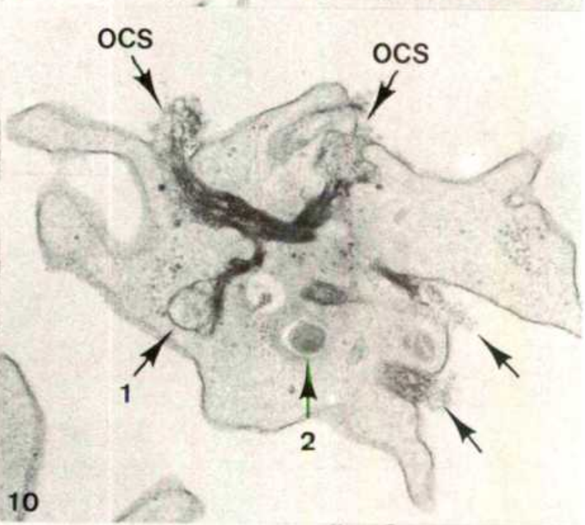
During the course of experiments on platelet clots,[47] it became apparent that tannic acid reacted with fibrin strands

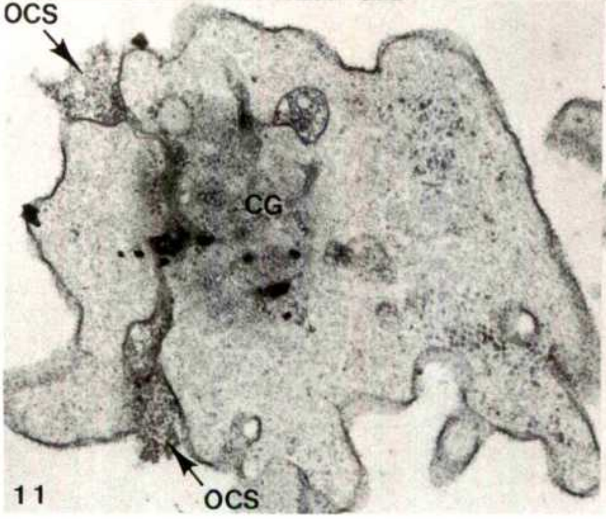
Tannic acid has resulted in heavy staining of the fibers by osmic acid (fixation: Glut-TA-OsA). Magnification × 33,000. Fig 3. Platelet from a washed suspension exposed to 5 U/mL of thrombin for three minutes and fixed in solutions containing tannic acid. Intensely stained fibers (F) identical to fibrin strands formed in C-PRP and fibrinogen solutions exposed to thrombin and stained with tannic acid were evident in swollen granules and channels of the OCS (fixation: Glut-TA-OsA). Magnification × 17,000. Fig 4. Platelet aggregate from sample of C-PRP exposed to 1 U/mL of thrombin for three minutes. Clumps of tannic acid–osmium stained fibrinogen are evident around and between the aggregated cells. Some granules (1) are unstained while others (2) show a strong reaction. Another granule (3) communicates through dilated and narrow segments of the OCS with the surface membrane on the other side of the platelet. A fourth granule (4) appears to communicate with two segments of the OCS in an adjacent platelet (fixation: Glut-TA-OsA). Magnification × 25,000. Fig 5. Platelet from the same sample of aggregated platelets shown in Fig 4. Some granules (1) are unreactive while others (2) are intensely stained by tannic acid–osmium. Other granules (3) are swollen and their contents diluted. A channel of the OCS connects the third (3) organelle to the surface on the other side of the platelet. A dilated connection of the OCS to the surface is evident on the right side of this cell (fixation: Glut-TA-OsA). Magnification × 25,000. Fig. 6. Platelet from washed suspension exposed to thrombin at a concentration of 5 U/mL for one minute in the presence of EDTA. A channel of the OCS filled with granule contents follows a tortuous course from one side of the centrally concentrated contractile gel (CG) and constricted ring of microtubules (MT) to an opening on the opposite side. Tannic acid–osmium stained material appears to have been fixed in the process of extrusion from the opening (fixation: Glut-TA-OsA). Magnification × 29,000.











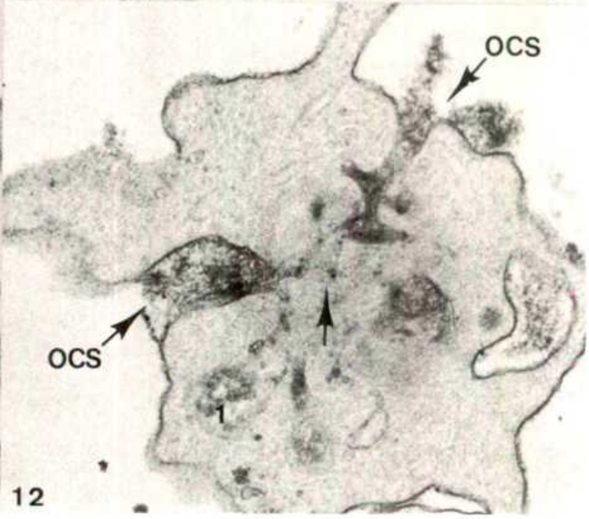


Fig 7. Platelet from EDTA washed suspension combined with thrombin at 1 U/mL for five minutes. A tortuous channel filled with tannic acid–osmium stained material passes from one side of the platelet to the other and opens on the surface. Granules (G) are connected to the channel which has dilated and narrow (↑) segments (fixation: Glut-TA-OsA). Magnification × 29,000. Fig 8. EDTA washed platelet exposed to 3 U/mL of thrombin for five minutes. Stained (1) and swollen (2) granules are directly connected to the channel filled with material reacting with tannic acid–osmium. Other swollen granules, such as (3), are connected to different channels of the OCS and pass out of the plane of section. Microtubules (MT) are constricted into the central mass of contractile gel (fixation: Glut-TA-OsA). Magnification × 29,000. Fig 9. Platelet from washed suspension containing EDTA and exposed to thrombin (3 U/mL) for three minutes. A complex channel of the OCS is imbedded in the central mass of contractile gel (CG). The channels are an interconnected spiderweb of

and caused intense staining by osmic acid. The observation prompted the studies carried out in this investigation. Tannic acid, when combined with PPP or solutions of purified fibrinogen in the same manner used during glutaraldehyde fixation, caused immediate formation of precipitates. The amorphous and fibrillar material present in the precipitates resembled the fibrinogen and fibrin in tannic acid–stained, fixed samples of resting or activated C-PRP. Addition of thrombin to plasma or fibrinogen solutions before tannic acid resulted in selective deposition of osmic acid on fibrin strands.

The present investigation has used this feature of tannic acid pharmacology to evaluate the release reaction in thrombin-aggregated and -activated platelets. Fibrinogen is a major constituent of platelet alpha granules and is extruded from activated platelets during release.[48] It seemed possible that tannic acid might combine with fibrinogen and selectively deposit osmic acid during transfer of the clottable protein from cytoplasmic organelles to the platelet exterior. Thrombin-aggregated platelets in PRP samples stained with tannic acid were identical to control aggregates, except for the osmium staining reaction. The amorphous, dense precipitate surrounding aggregates and concentrated in spaces between cells resembled the precipitate formed in solutions of fibrinogen after addition of tannic acid. A similar electron-dense material was evident in some of the platelet alpha granules and filling narrow and dilated elements of the OCS. Although alpha granules did not react with tannic acid in resting platelets, many normal-sized and swollen granules in aggregated cells were intensely stained by tannic acid–osmium. Filamentous material and strands filling dilated channels and swollen granules were identical in appearance to electron-dense fibers in thrombin-clotted fibrinogen solutions.

Involvement of granules and channels in the platelet secretory process was clearly shown in the study of individual cells. Washed platelets exposed to thrombin in the presence of EDTA did not reveal the amorphous electron-dense precipitate outside the cells as seen in thrombin-aggregated C-PRP because nearly all of the fibrinogen was removed during preparation and resuspension. The internal platelet changes, however, were essentially identical. Thrombin stimulation caused shape change, central movement of organelles, and constriction of microtubule coils and microfilaments into a contractile gel. Whereas granules and lumina of OCS channels were unstained in resting platelets fixed in the presence of tannic acid, osmium-reactive material filled the dilated OCS of thrombin-activated platelets and stained granules attached to the channels intensely. Other granules and organelles not in continuity with the OCS remained unstained. The appearance suggested that tannic acid reaches the interior of granules by traversing channels of the OCS, rather than by diffusing through cell membranes and cytoplasm.[44] The reaction to thrombin breaches the membrane barrier between channels and granules so that they are in direct continuity. Many proteins are secreted from platelet alpha granules,[48] and tannic acid, as used in this study, may combine with them. Yet, our studies show that tannic acid reacts with fibrinogen and fibrin, and selectively deposits osmium. As a result, it is reasonable to suggest that tannic acid identifies the secretion of fibrinogen from granules to channels, and through connections with the cell surface to the exterior of thrombin-activated platelets.

Evidence favoring an alternative secretory pathway involving fusion of centrally concentrated granules to each other, formation of a closed vacuole filled with substances from the organelles, migration to the surface, attachment to the cell wall, and discharge of contents to the outside[12,13] was not encountered in the present study. The possibility that centrally concentrated granules might fuse with each other at an early stage in the secretory process has been attractive for many years and was suggested in early investigations.[49] That granules can fuse with each other has been demonstrated in studies of platelets from individuals with inherited and acquired platelet disorders.[50-52] More concrete evidence has been provided by investigations of platelet morphology during long-term storage in vitro.[53] Fusion of normal-sized organelles to form giant granules is a common finding after storage under mildly alkaline conditions for seven to 21 days. A stage of fusion similar to that observed in long-term–stored platelets would seem essential in formation of closed secretion vacuoles. Although the appearance of vacuoles in thrombin-activated platelets has frequently been reported,[10,14] none of the studies has shown that fusion of granules into a membrane-enclosed mass precedes development of empty sacs. Apparent fusion of two granules was reported in a previous study of platelet secretion employing tannic acid.[54] In our experience, however, such an event is extremely rare.

A possible explanation for development of vacuoles in thrombin-treated platelets is provided by the present study. After establishing communication with channels of the OCS, granules become swollen. Often several granules are attached to a single channel. Dilatation of the channel and

canaliculi. Segments of cytoplasm (C) often appear isolated by the interconnected canaliculi, which dilate as they fill with granule contents. Electron-dense material appears to be in the process of extrusion from this cell (fixation: Glut-TA-OsA). Magnification × 29,000. Fig 10. Platelet from sample of EDTA washed platelets exposed to thrombin at 3 U/mL for one minute. Electron-dense material is in the process of extrusion from four different sites (↑) on the surface of this cell. One of the channels inside the cell communicates with two different openings of the OCS and with a granule (1). Other granules (2) are not stained by tannic acid–osmium (fixation: Glut-TA-OsA). Magnification × 29,000. Fig 11. EDTA washed platelets combined with 5 U/mL of thrombin for five minutes. A third example showing a channel of the OCS opening at two different sites on the platelet surface. They demonstrate clearly that granules do not form closed sacs that migrate to and fuse with the cell surface. Transplatelet channels communicating with opposite surfaces of the same cell are inconsistent with that concept (fixation: Glut-TA-OsA). Magnification × 29,000. Fig 12. EDTA washed platelets following exposure to thrombin at 3 U/mL for three minutes. Another example in which a channel stretches from one side of the platelet to the other side and opens at two separate sites on the cell surface. One granule (1) connects directly to the transplatelet channel. The central portion of the canaliculus (↑) is less intensely stained, but can easily be identified (fixation: Glut-TA-OsA). Magnification × 29,000.

attached granules would and does yield the appearance of a large vacuole. The channels often appear dilated in the cytoplasm, but narrow at the cell surface.[54,55] Swelling of granules and channels with persistent narrowing of the OCS opening would produce the appearance of a distended vacuole close to the cell wall when viewed in a fluorescence microscope after staining with a monoclonal antibody to a specific granule constituent.[12,13]

In conclusion, the present study has reexamined involvement of the surface-connected, open-canalicular system in secretion by human platelets stained with tannic acid following exposure to thrombin in the presence or absence of EDTA. Findings of the investigation confirm earlier studies which suggested that channels of the OCS were major conduits for discharge of products secreted during the human platelet release reaction. Granules are concentrated in centers of thrombin-activated platelets, but fusion of the organelles to each other resulting in formation of closed sacs was not observed. Instead the sequence of granule interaction with channels, establishment of continuity, swelling of channels and granules as secretory products are exposed to the medium, and discharge through narrow and dilated openings of OCS on platelet surfaces was clearly demonstrated by fixation in the presence of tannic acid. Efforts to apply this procedure to platelets from a patient with congenital afribrinogenemia are currently in progress.

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The Syndrome of T8 Hyperlymphocytosis: Variation in Phenotype and Cytotoxic Activities of Granular Cells and Evaluation of Their Role in Associated Neutropenia

By Catherine Grillot-Courvalin, Giovanna Vinci, Andreas Tsapis, Marie-Christine Dokhelar,
William Vainchenker, and Jean-Claude Brouet

We performed a longitudinal study of the phenotype and functions of granular lymphoid cells from seven patients with T_8 hyperlymphocytosis and neutropenia. Whereas cells retained a T3+ T8+ (six cases) or T3− T8+ (one case) phenotype at different examinations, the expression of natural killer (NK)-related antigens (HNK1- and Leu11-defined antigens) exhibited striking variations, some of which were also observed after in vitro culture. Similarly, natural or antibody-mediated cytotoxic activities fluctuated in vivo and in vitro. Cells from the patient with T3− T8+ proliferation were able to inhibit directly the growth of early CFU-GM, CFU-E, and BFU-E and to a lesser extent of late CFU-GM, as shown by cultures of autologous blood or marrow progenitors after depletion (and subsequent addition) of granular cells. In the other six patients with T3+ T8+ cells, no such effect was found. However, after a 24-hour incubation of the progenitors with the granular cells, CFU-GM growth was clearly inhibited; this was not observed in all experiments, a finding which may be related to the spontaneous variations of cell killer functions. Finally, no correlation was noted between the clinical course or extent of lymphoid proliferation and cell function or phenotype or with the monoclonal (two cases), polyclonal (three cases) or germ-line (one case) patterns of T cell receptor β gene configuration.

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RECENTLY, THE ASSOCIATION of chronic T8 hyperlymphocytosis and neutropenia has been the subject of several reports, since it represents a distinct new clinical, morphological, and immunological disorder, the pathogenesis of which is still largely unknown.[1-4] Because of their strong similarity to large granular lymphocytes (LGL) and the occurrence of cytopenia, T8 cells from these patients have been studied mainly for killer and suppressive activities, with contradictory results. These cells frequently display antibody-dependent cell cytotoxicity (ADCC),[3,5-7] whereas evaluation of natural killer (NK) activity or suppression on Ig synthesis or hemopoietic cell maturation yielded opposite results in different studies.[3,6-15]

We report here striking variations of the cell surface phenotype and/or functions in a longitudinal study of seven patients with T8 hyperlymphocytosis and neutropenia. Some of these modifications also occurred after short term in vitro cell cultures. These unexpected variations may be germane to the above-mentioned discrepancies and to the difficulty encountered in understanding the physiopathology of this disorder. Studies in six cases of T cell receptor β chain gene organization showed unrearranged, polyclonal, or monoclonal patterns without clear-cut correlations with clinical data or course of the disease.

MATERIALS AND METHODS

Patients. Seven patients with T8 hyperlymphocytosis and neutropenia were studied. The main clinical and hematologic data are listed in Table 1. Their clinical condition remained stable during a two- to 18-year follow-up period. There were no life-threatening infections in any patient, although recurrent upper respiratory infections occurred in five patients. In all cases, the majority of blood lymphoid cells were medium- or large-sized granular cells with azurophilic granules and strong acid phosphatase positivity. Peripheral thrombocytopenia as assessed by a reduced half-life of autologous ^{51}Cr-labeled platelets (two and three days for patients 6 and 4, respectively) was observed in two patients. Ferrokinetic studies were performed in the two anemic patients. In patient 1, there was a mild autoimmune Coombs test–negative hemolysis with a splenic sequestration. In patient 5, there were both a low production and abnormal maturation of red cells.

A definite bone marrow lymphoid infiltration was observed in four patients at biopsy; the granulocytic series appeared normal or slightly hypoplastic. Monoclonal Ig were detected in two patients: patient 1 had a γ 3 heavy chain disease protein with bone marrow and spleen plasmacytic infiltration; a monoclonal IgG lambda was found in serum of patient 3. No Ig abnormalities were observed in the other patients.

Cell isolation and phenotype. Mononuclear cells were isolated from peripheral heparinized blood or bone marrow by centrifugation on Ficoll Hypaque density gradients. In patient 1, spleen cells were obtained by gentle teasing of the organ. In some experiments, E-rosetting cells were isolated and rosetting lymphocytes recovered after hypotonic lysis of sheep red cells; in some preparations, T8 cells were eliminated by complement-dependent cytotoxicity.

The cells were studied with a panel of monoclonal antibodies (Mab) by indirect fluorescence using fluoresceinated goat IgG to mouse Ig as a second layer. Anti-T cell Mab were directed against T3, T4, T8, T6, and Tac (IL2 receptor) antigens. Mab directed to LGL included HNK1 (Leu 7), Leu 11, NKH1, and NKH2.[16] K5 Mab directed to class II DR antigens was produced in our laboratory. B cell surface Ig were detected by direct fluorescence with monospecific, rhodamine-conjugated Fab'2 fragments of rabbit IgG to human heavy and light Ig chains. Cell surface phenotype was determined on fresh cells and in certain cases after three days in vitro culture in RPMI 10% fetal calf serum (FCS) medium with or without addition of 10% T cell–conditioned medium (a supernatant of T cells stimulated for 48 hours with phytohemagglutinin).

Functional studies. Natural killer and ADCC assays: The tar-

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Table 1. Main Clinical Symptoms in Seven Patients with T8 Lymphocytosis

	Patients						
	1	2	3	4	5	6	7
Absolute lymphocytosis 10^9/L	10	5	6	4.5	8	10	14
Neutrophils 10^9/L	0.1	0.5	0.4	0.7	0.5	0.3	0.5
Anemia (Hb < 10 g/L)	+	–	–	–	+	–	+
Thrombocytopenia (<100 × 10^9/L)	–	–	–	+	–	+	–
Lymph node enlargement*	–	–	–	–	–	+	–
Splenomegaly*	+ +	–	–	–	–	+	+ +
Bone marrow							
lymphoid infiltration on biopsy	+	–	–	+	+	+	–
% lymphoid cells on smear	25	8	12	25	15	50	8
Recurrent infections	+	–	–	+	+	+	+

* +, slight; + +, moderate.

get cells used for NK assay were the erythroleukemic K562 cells and for ADCC L1210 leukemic cells sensitized by rabbit anti-L1210 antibodies. ^{51}Chromium-labeled cells and patient cells were incubated in triplicate at various effector/target ratios in microtiter plates for four hours at 37°C and 100 μL of cell free supernatants were collected and counted for ^{51}Cr release in a γ counter. The percentage cytotoxicity was calculated as follows: % cytotoxicity: (experimental release – spontaneous release) × 100/maximum release – spontaneous release.

Clonogenic assays. Marrow or peripheral blood mononuclear cells were obtained after informed consent; they were cultured, usually in triplicate, using the plasma clot technique.[17] The final medium consisted of alpha medium (Eurobio, Paris) 10% human AB serum, 1% deionized bovine serum albumin, 70 μg/mL $CaCl_2$ and 10% bovine citrated plasma. Several stimulating factors for CFU-GM growth were used, ie, Mo-,[18] 5637-,[19] and GCT[20]-conditioned media and phytohemagglutinin leukocyte conditioned medium. In some experiments, erythroid colonies were grown by adding 1 U/mL porcine erythropoietin (CNTS, Paris), to 5% Mo-conditioned medium. At day 12 to 14 CFU-GM- or BFU-E-derived colonies were enumerated under an inverted microscope; CFU-E colonies were counted at day 7.

Blood mononuclear cells depleted of T cells were plated at 5 × 10^4 to 2 × 10^5 cells/mL. Purified E-rosetting T cells were added at different concentrations (1 × 10^5 to 1 × 10^6/mL) to the non-T cells obtained from the patient or normal individuals. In other experiments, blood or marrow mononuclear cells depleted of T cells, T cells alone, or a mixture of mononuclear cells depleted of T cells and T cells in a 1/4 ratio, were incubated overnight at 5 × 10^5/mL in alpha medium 10% FCS at 37°C. Cells were then centrifuged and counted. Individual populations of cells or mixed populations were assayed for colony formation. In some experiments sera of the patients were added at 10% final concentration.

Analysis of T cell receptor beta chain genes. High-molecular weight DNA was extracted from blood mononuclear cells and from patient 1 spleen T8 cells. DNA was digested with the restriction enzymes *Bam*HI, *Eco*RI, and *Hind*III and fractionated on 0.8% agarose gels. DNA fragments were transferred by the Southern process to nitrocellulose paper. Hybridization with ^{32}P-labeled probe for the C β constant gene of T cell receptor (400bp *Bgl*I-*Bgl*I fragment containing human C β gene sequence) was performed, followed by washing at the appropriate stringency (0.1 × SSC, 0.2% SDS, 65°C) and radioautography.

RESULTS

Cell phenotype. The majority of T cells from six patients formed E-rosettes and expressed both T3 and T8 antigens (Table 2); the density of T8 antigen often appeared quite low by eye fluorescence in comparison to that of normal blood cells; however, this phenotype remained stable at different examinations and after in vitro culture in RPMI FCS medium. The majority of these T3+ T8+ cells did not express class II antigens. The cells of patient 7 formed E-rosettes but expressed only T8 antigen. Four different markers of LGL were studied; the expression of HNK1 antigen was constant, although the percentage of positive cells changed strikingly in the same patient at different times (Table 2) or after three days of in vitro culture in patient 2. Cells from only five patients were stained with Leu 11 Mab, which reacts with a Fc receptor-related epitope; again, large variations in the percentage of positive cells were noted in patients 4 and 6 in different experiments; Leu 11 expression was lost after in vitro culture in all cases studied (Table 2). Neither before nor after in vitro culture did the cells express two other NK cell-related antigens, identified by NKH1 and NKH2 Mab, which react with the majority or a subset of blood LGL, respectively. Mab directed to IL2 receptor (Tac) strongly stained 10% of T3+ T8+ cells in the spleen of patient 1 and 2% of blood cells in patient 3, whereas it was negative on peripheral blood leukocytes (PBL) from the other patients.

Killer function. Since the above results showed variations in cell phenotype at different times, we performed a longitudinal study of ADCC and NK activity in 6 patients.

PBL from patients 2 and 5 displayed a normal ADCC function in the single assay performed. Cells from patient 1 exhibited similar ADCC levels (50% Cr release as compared to normal PBL) on two separate tests. Divergent results were observed in patient 3, who was studied twice at six-month intervals (normal *v* absent ADCC).

NK function of freshly drawn PBL was studied during a 2-year period (Table 3). In patients 1, 4, and 5, NK activity was abnormally low or absent. Discordant results (normal *v* low activity) were obtained in patients 2, 3, and 6 in separate examinations. NK activity of spleen cells from patient 1 was studied; unseparated cells, purified T cells, and T4-depleted T cells exhibited a similar killer function, ascertaining therefore that the T8 subset mediated this effect. Unexpectedly, a fair NK activity was recovered after 48-to 72-hour in vitro culture in RPMI-FCS medium in all patients tested (Table 3). This activity was slightly enhanced when cells were

Table 2. Phenotype of Blood (or Spleen) Cells

	T Antigens*						LGL Antigens*				
	T3	T4	T8	T6	Tac	E-Rosettes†	HNK 1	Leu 11	NK 1A	NK 1B	DR Antigen*
Patient 1											
Spleen	88	14	67	0	10	80	57	1	1	1	13
Exp 1	—‡	2	80	—	1	85	2	—	—	—	9
Exp 2	88	10	77	0	0	—	14	—	—	—	10
Patient 2											
Exp 1	90	1	62	0	—	85	4	—	—	—	12
Exp 2	90	15	50	—	0.1	88	43	4	2	0.1	10
Exp 2§	85	7	60	—	0.1	—	0.5	2	0.2	0.2	—
Patient 3											
Exp 1	85	11	50	—	2	55	12	28	1	0.1	15
Exp 1§	—	—	56	—	0.5	—	22	0.1	1	1	—
Exp 2	90	10	65	—	—	65	25	30	1	1	12
Patient 4											
Exp 1	90	12	77	0	0	91	26	45	1	0.1	16
Exp 1§	—	—	80	—	0	—	27	9	1	0.1	—
Exp 2	85	5	95	—	—	85	70	20	1	1	12
Patient 5											
Exp 1	80	2	80	0	0	85	1	—	—	—	—
Exp 2	90	18	71	—	0	80	40	70	1	0.1	14
Exp 2§	—	—	75	—	0	—	34	2	1	0.1	—
Exp 3	—	—	80	—	—	85	50	—	—	—	8
Patient 6											
Exp 1	88	8	80	0	—	85	20	0	1	1	10
Exp 2	90	4	50	—	0	—	3	30	1	1	7
Patient 7											
Exp 1	5	3	30	0	1	80	40	95	1	1	3

*Monoclonal antibody–defined cell phenotype (indirect fluorescence; percentage of positive cells).

†Percentage of cells forming E-rosettes.

‡Not done.

§Phenotype after 72-hour in vitro culture in RPMI-FCS medium.

cultivated in phytohemagglutinin-conditioned medium (data not shown). These modifications were not related to an expansion of residual NK cells with a full phenotype of LGL, since phenotyping of the cultured cells did not disclose a sizable number of NKH1- or NKH2-reactive cells.

Clonogenic assays. In the first six patients, CFU-GM from blood cells were normal or quite high in patients 5 and 6 and markedly diminished in the others. Assays were repeated two to four times during a 2-year period and no marked difference in the plating efficiency was observed. Similar results were obtained whatever the stimulating factor used. The effect of T cell depletion by E-rosetting and of their subsequent addition was studied in all patients (Table 4). Depletion of T cells did not enhance colony formation and accordingly their addition did not decrease the number of colonies but even led to enhancement of colony formation in some experiments. Addition of T cells even at a very high concentration (up to 1 × 10^6 cells/mL) had no inhibitory effects. Results using marrow cells were identical (Table 5). In order to exclude that after E-rosetting, a small amount of remaining T3+ T8+ cells could still inhibit CFU-GM colony formation in patients with a low plating efficiency, T8 complement-mediated cytotoxicity was performed on T-depleted blood mononuclear cells from patients 2 and 4 and on T-depleted marrow cells from patient 2. Colony formation was not enhanced and addition of T cells did not alter the growth of progenitors. T cells from all patients were cultured with allogeneic normal marrow cells; no inhibition of colony formation was observed (data not shown).

When T cells were incubated for 24 to 48 hours in liquid medium and then added back to T-depleted mononuclear cells, no inhibition of colony growth in three patients was observed. In contrast, in patients 2, 5 and 6, a marked inhibition of CFU-GM colony formation was observed when T cells and T-depleted blood or marrow mononuclear cells were incubated together and then plated in semisolid medium (Table 6). This result was obtained both when using blood or marrow cells as the target for patient 2. However, this result was not observed in all experiments; a marked inhibition was detected in three of five different occasions during an 18-month period for patient 2, and once in two experiments for patients 5 and 6. Sera from all patients were tested in culture (and in preincubation experiments in three patients); no inhibition of colony formation was noted. Studies of CFU-E colonies were performed in patient 5, who was anemic. T cell–depleted cultures yielded a normal number of colonies (260 colonies/10^5 cells); addition of PBL T cells clearly suppressed CFU-E growth (32% inhibition, $0.05 < p < 0.01$).

Similar experiments were performed in patient 7 whose cells had a T3− T8+ phenotype. Studies of blood cell progenitors (Table 4) showed that depletion of T cells by E-rosetting allowed the growth of CFU-GM, whereas their subsequent addition inhibited colony growth. However, no

Table 3. Natural Cytotoxicity of T8 Cells on K562 Target

	Effector/Target Ratio				
	50:1	25:1	10:1	5:1	2:1
Patient 1					
Exp 1	14.5*	7	2	0.5	0
Exp 2	6	2	0.2	—	—
Exp 3†	34	13	3	—	0
Patient 2					
Exp 1	35	19	14	—	—
Exp 2	0	0	0	0	0
Exp 2‡	50	40	21	12	4.5
Patient 3					
Exp 1	4	1	1	0.6	1
Exp 2	81	62	42	—	—
Exp 3	28	13	1	—	—
Exp 3‡	81	41	25	12	7
Patient 4					
Exp 1	15	8	4	1.5	0.4
Exp 2	10	1	0	0	0
Exp 2‡	39	23	9	5	2
Patient 5					
Exp 1	3	2.5	1	0.1	—
Exp 2	0	0	0	0	0
Exp 2‡	19	6	2	0.5	0
Patient 6					
Exp 1	15	—	5	—	1
Exp 2	60	46	26	1	0
Patient 7					
Exp 1	66	34.7	25.7	12.3	1.7

*Percentage of killing.

†Spleen cells.

‡NK activity of cells after three-day in vitro culture in RPMI-FCS medium (the phenotype of cells before and after in vitro incubation is shown in Table 1).

clear effect was observed on marrow CFU-GM–derived colonies (Table 5). In addition E+ cells were able to significantly inhibit marrow CFU-E and BFU-E growth ($p < 0.01$); however, they still had no definite effect on marrow CFU-GM growth. When E+ cells from this patient were incubated for 24 to 48 hours with T-depleted marrow or blood cells, inhibition of blood CFU-GM, CFU-E, and BFU-E colony formation was again observed, but no significant effect on marrow CFU-GM colony formation could be recorded.

Table 4. CFU-GM Colony Formation From Blood Cells (Day 14)

Patient	Mononuclear Cells (10^6)	T-Depleted Mononuclear Cells	E$^-$ and T Cells
1	12 ± 4	9 ± 4	8 ± 3
2	9 ± 3	9 ± 2	19 ± 8
3	14 ± 3	11 ± 2	12 ± 3
4	3 ± 1	3 ± 2	3 ± 1
5	350 ± 40	378 ± 45	385 ± 30
6	56 ± 9	62 ± 8	63 ± 7
7	0	23 ± 2	3 ± 1

T cells were depleted by E-rosetting. E$^-$ and E$^+$ were recovered and counted. E$^-$ were seeded at a concentration equivalent to their presence in 1 × 10^6 nonseparated mononuclear cells. The number of E$^-$ was usually 1 × 10^5 cells/mL. E$^+$ were added at a different concentration which did not change the plating efficiency. Results reflect the addition of E$^+$ in 1/9 ratio.

Table 5. CFU-GM Colony Formation From Marrow Cells (Day 7)

Patient	Mononuclear Cells (10^5 Cells/mL)	T-Depleted Cells	E$^-$ and T Cells at a ⅑ Ratio
1	50 ± 10	25 ± 4	53 ± 8
2	96 ± 20	90 ± 15	93 ± 18
5	ND	485 ± 32	494 ± 8
7	ND	200 ± 14	196 ± 14

Experiments were performed as detailed in Table 4, except that colonies were enumerated at day 7.

ND, not determined.

Analysis of T cell receptor beta chain gene organization. DNA from all cases except patient 2 was studied for T cell receptor beta chain gene configuration. In patients 5 and 6, a monoclonal rearrangement was observed after digestion of DNA with *Bam*HI and *Hind*III; with *Eco*RI the 11-Kb band had a markedly decreased intensity, but a rearranged band was detectable only in patient 5 (Fig 1). In patient 7, beta chain genes were in germ-line configuration. In the three remaining patients, no rearranged band was detected after DNA digestion with the three enzymes (Fig 1). A decrease of the intensity of germ-line bands in these cases is indicative of polyclonal T cell population.

DISCUSSION

The seven patients studied were affected with the recently characterized syndrome of T8 hyperlymphocytosis with neutropenia. Their disease followed a chronic course characterized by hyperlymphocytosis (which spared bone marrow in three cases), neutropenia, and frequent, mild infections.

Two patients had monoclonal Ig, a hitherto-unreported finding in such patients. Patient 1 had a $\gamma 3$ heavy chain disease protein with plasmacytic infiltration of spleen and bone marrow, and patient 3 a $\gamma\lambda$ monoclonal Ig that was detected after three-year follow-up and remained stable thereafter. Although such Ig abnormalities appear distinctly unusual, the occurrence of polyclonal hypergammaglobulinemia as well as various autoantibodies are frequent in this syndrome.[3,13,14] Whether these abnormalities are somehow related to the T cell disorder is presently unknown.

In all patients, the cells had the morphology of medium-sized or large lymphocytes with azurophilic granules. In one

Table 6. CFU-GM Formation After 24-Hour Preincubation of E$^-$ Cells with E$^+$ Cells

	E$^-$ Cells	E$^-$ E$^+$ Cells	E$^+$ Cells
Patient 2			
Exp 1*	42 ± 2	25 ± 5	2 ± 1
Exp 2*	42 ± 6	19 ± 5	0
Exp 3†	30 ± 2.5	9.5 ± 2	1 ± 1
Patient 5			
Exp 1*	72 ± 16	28 ± 6	0
Patient 6			
Exp 1*	22 ± 1	14 ± 1	0

E$^-$ and E$^+$ cells prepared as described in Material and Methods were incubated for 24 hours before plating for CFU-GM assay. Results are shown for experiments where a clear inhibition was observed.

*Peripheral blood mononuclear cells.

†Bone marrow cells.

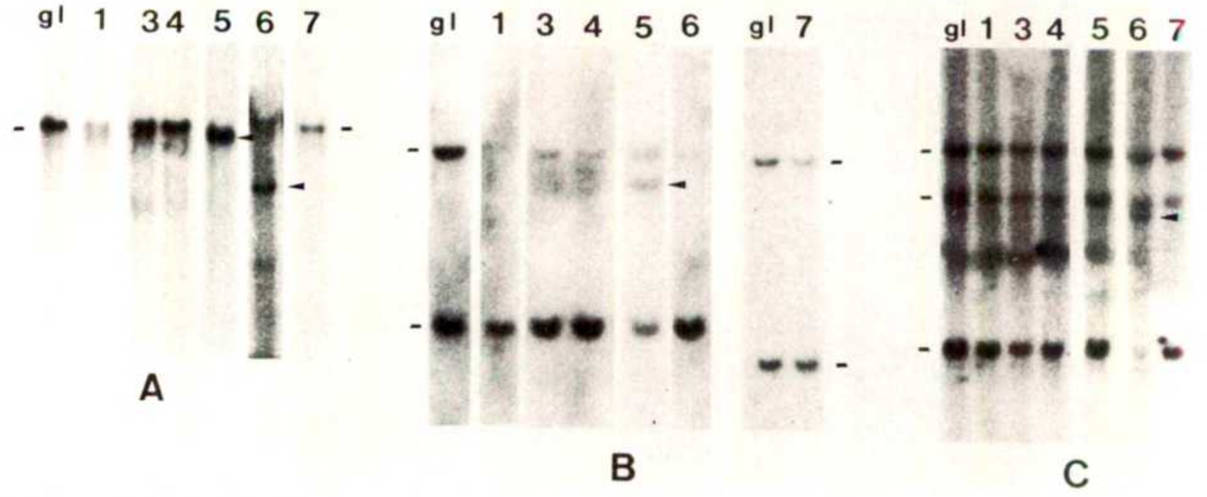

Fig 1. Southern blot analysis of DNA fragments from six patients with T cell receptor gene probe. Abbreviation: gl, germ line placental DNA. Numbers refer to patients (see Table 1). DNA was digested with *Bam*HI (A), *Eco*RI (B) and *Hind*III (C) restriction enzymes. Horizontal bars indicate germ line fragments; arrows, rearranged fragments.

case (patient 7) cells were devoid of T3 antigens, possessed germ-line T cell receptor beta chain genes and expressed some NK cell–related markers (HNK1 and Leu 11, reactivity) as well as T8 antigen. In the other six cases, they belonged unequivocally to the T cell series as shown by the expression of T3 and T8 antigens and by the configuration of T cell receptor beta chain genes. They also possessed some of the known markers of LGL, namely HNK1 (seven cases) and Leu 11 (five cases). However, only a percentage of T3+ and T8+ cells bore these antigens and longitudinal studies showed striking variations of the subset of cells expressing detectable levels of these NK-related antigens; some of these variations could be observed after in vitro culture. These modifications contrasted with the stable T3+ T8+ phenotype of the majority of PBL of blood cells and do not support the suggestion that there is significant heterogeneity within the expanded lymphocytic population.[8] Moreover, in patient 1, the percentage of HNK1 reactivity among the T3+ T8+ cells was different in the blood or spleen. The constant absence of NKH1 and NKH2 markers which identify the majority of functional NK cells (or subsets) argues against the possibility that the observed variations are related to a T3− normal residual LGL population; it indicates furthermore that the T3+ T8+ HNK1+ cells characteristic of this syndrome belong to a distinct population of NK cells. This finding therefore reinforces the rationale to characterize the syndrome of T8 hyperlymphocytosis with neutropenia.

More importantly, this longitudinal study also revealed variations in both natural and antibody-dependent killer activities. The significance of these phenotypic and functional fluctuations remains uncertain; interestingly, they were also recorded in primary and transplanted tumors of the rat LGL leukemia.[21] NK function was restored after in vitro culture but we could not find any serum inhibitor, as suggested for normal LGL[22] and in another case of T8 hyperlymphocytosis.[23] Anyway, our data are probably germane to the discordant results previously published.

The benign or malignant nature of this syndrome is still controversial. Cytogenetic studies as well as the clinical course[13,14,24-26] undoubtedly suggest that at least some patients have an indeed malignant disease; no clinical or immunological data, however, delineate this subset of patients. Investigation of clonal rearrangements of T cell receptor β chain genes of the expanded population of granular lymphocytes does not resolve this issue. In our patients with T3+ T8+ phenotype a clonal rearrangement was observed in two cases, while a polyclonal pattern was noted in three other patients as shown by a decreased intensity of germ-line bands and the absence of rearranged DNA fragments. In the patient with T3− T8+ cells, T cell receptor genes appeared in the germ-line configuration. Similar studies have been performed in 23 other patients with T8 hyperlymphocytosis and neutropenia[27-30]; clonal rearrangements were noted in 17 cases and polyclonal or germ-line pattern in the other cases. In one large study, only T3− T8+ cells had germ line pattern whereas all T3+ T8+ cells appeared monoclonal.[29] The absence of detailed cell phenotypes in the other reported cases with unrearranged pattern precludes definite conclusions about the incidence of polyclonal T3+ T8+ hyperlymphocytosis. It should be stressed that in our series no clinical, hematological, or immunological features, neither duration of the disease nor bone marrow or spleen lymphoid involvement, distinguish our patients with clonal disorder. The rather uniform clinical and immunological data (and especially the discrete NK-related phenotype as outlined above) may suggest a polyclonal expansion of a subset of NK-related cells, driven by an unknown event or secondary to the neutropenia, with the possibility of a late clonal evolution. The derivation of NK cells is still debatable. From the above data it can be hypothesized either that the two phenotypes (T3− T8+ and T3+ T8+) correspond to different lineages, or that T3− T8+ cells with high NK activity represent precursors of T cell lineage.

It should be noted that the existence of polyclonal T3+

T8+ cells as well as that of T3− T8+ hyperlymphocytosis does not support the possibility that the neutropenia is due to an interaction involving the conventional T cell receptor. Indeed the role of granular lymphoid cells in the genesis of cytopenia in these patients is still uncertain. In previous reports, a consistent inhibition of colony-forming cells has been noted only for erythroid progenitors in patients with anemia.[3,12] In contrast, most studies failed to demonstrate any effect of expanded granular cells on granulopoiesis, even when the cells had some suppressive properties on cell proliferation or Ig synthesis by B cells in vitro.[1,3,6,14]

We reevaluated here the possible interaction of T8+ cells with myeloid progenitors using a number of assays. Two different situations could be distinguished. First, cells from patient 7 with T3− T8+ phenotype directly interfered with hemopoietic progenitors; depletion of T8+ cells led to a striking increase of both early CFU-GM and BFU-E; likewise, addition of the purified granular cells inhibited early CFU-GM, CFU-E, and BFU-E but had no effect on late CFU-GM colonies. This activity is reminiscent of that exerted by normal LGL,[31-34] although studies of inhibitory effects of LGL on hematopoietic cells led to some discrepancies, possibly because of technical conditions. Like normal LGL, granular cells from this patient had unrearranged T cell receptor β chain genes; whether their effect is mediated by direct contact or production of colony inhibiting factor as shown for normal LGL[32] remains to be established.

Studies performed in the six other patients with T3+ T8+ cells were in sharp contrast with the latter results. No modification of colony growth was observed after depletion (and subsequent addition) of granular T3+ T8+ cells in 18 experiments performed in the six patients. These patients had either a normal colony growth (two cases) or a reduced one (four cases), a reproducible finding at different examinations. Therefore, we obtained no evidence that cells directly suppress hemopoiesis. These cells are obviously different from the suppressive T cell population, which has been shown to inhibit with a class II restriction hemopoiesis in normal subjects[35-37] or in disease states,[36,37] and which feature a T8+ HNK1+ T3+ DR+ phenotype.

Cultures in autologous serum yielded similar results, making unlikely a role for antineutrophil antibodies that were present in the serum of four patients as noted in some reports[5,14] but not others.[3,6] However, a role for ADCC is not ruled out by our experiments if sensitized progenitors are unavailable to study because of in vitro destruction.

We next explored the possibility that T3+ T8+ cells from these patients need to interact with progenitors or some kind of activation to become suppressive. Indeed, the finding in patient 1 of T3+ T8+ cells expressing IL 2 receptors in spleen but not blood may indicate that suppressive cells do not circulate and may be locally activated. In fact, preincubation of T cells with blood or marrow progenitors led to significant inhibition in three of six patients. However, this inhibition was not observed in all experiments (three of five assays in patient 2 and one of two in patients 5 and 6). Such variations could well be related to the changes occurring in phenotype and function of T8 cells as demonstrated in this report. Interestingly, such preincubation is considered necessary for normal LGL cells to act on hemopoiesis in some reports.[31,32]

Finally, although we cannot exclude the possibility that T cells do not play a major role in neutropenia in these patients, it is worth noting that patients 1 and 5 recovered a normal WBC and differential count after a 6-month chemotherapeutic regimen (intermittent prednisone, vincristine, and cyclophosphamide) given for associated lymphoid malignancy and marked anemia, respectively. Likewise, there was a clearing of the expanded T3+ T8+ cell population with normal ratio of T4/T8 cells.

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Role of Colony-Stimulating Factor in Myelopoiesis in Murine Long-Term Bone Marrow Cultures

By D.A. Lipschitz, K.B. Udupa, J.M. Taylor, R.K. Shadduck, and A. Waheed

Weekly medium change or midweek feeding of long-term bone marrow cultures (LTMBCs) results in a significant increase in total myeloid cell production. Proliferative myeloid cells peak 48 hours after feeding, and nonproliferative myeloid cells reach maximum levels at 72 hours. This increase in myelopoiesis is invariably preceded by a significant elevation in biologically and immunologically measurable colony-stimulating factor (CSF) in the supernatants of LTBMC. The level peaks 24 hours after medium change, then gradually returns to basal values. The decrease in CSF relates to its consumption by generating myeloid precursors because no fluctuation in the levels occur in cultures without active myelopoiesis. No significant inhibitors or promoters of CSF were detected. When highly purified L cell CSF, CSF in lung-conditioned medium, or CSF concentrated from LTBMC supernatant is added to cultures, an identical increase in myelopoiesis occurs. Anti-CSF antiserum, added to culture at the time of medium change, totally neutralizes supernatant CSF levels but does not affect myelopoiesis. These findings suggest a potential regulatory role for CSF in myelopoiesis in LTBMC. CSF appears to function within the microenvironment through a mechanism involving cell:cell interactions or by causing the production of other substances that stimulate myelopoiesis. Because exogenous CSF stimulates myelopoiesis, it is likely that it too can react either directly or through microenvironmental cells to stimulate primitive myeloid cells to divide.

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RECENTLY tissue culture techniques have been developed that permit long-term maintenance of bone marrow cells in vitro for up to 50 weeks.[1-3] The key component of the culture system is the formation of an adherent microenvironmental layer that allows proliferation and differentiation of hematopoietic stem cells into predominantly mature myeloid precursors. These myeloid cells become nonadherent and can be harvested at weekly intervals from the culture medium. The cellular and morphological characteristics of hematopoiesis in long-term bone marrow culture (LTBMC) bear many similarities to normal hematopoietic function in vivo. This culture system has been extensively used to determine the role of colony-stimulating factor (CSF) in the regulation of myelopoiesis. Early work failed to detect measurable CSF in culture, and other studies demonstrated no increase in myelopoiesis when CSF from different sources was added to culture. These included CSF contained in lung-conditioned medium (LCM), WEHI-3 cell-conditioned medium, and L cell CSF condition medium.[4,5] Furthermore, addition of anti-CSF antiserum had no apparent effect on myeloid cell production. This led to the suggestion that myelopoiesis in LTBMC was independent of CSF or alternatively, that CSF production was minimal and functioned exclusively locally. More recent studies provided stronger evidence indicating a direct regulatory role of CSF in myelopoiesis in LTBMC. Using a bilayer agar culture system, Heard and colleagues were easily able to detect significant amounts of CSF production in LTBMCs.[6] A relationship between myeloid cell production and CSF levels was suggested by the very high levels of CSF found in hematopoietically inactive cultures and a marked decrease in its level when these adherent layers were recharged. Furthermore, an inverse correlation between supernatant cell recovery prior to the assay and the level of CSF was noted. Gualtieri and co-workers provided further support for the importance of CSF in myelopoiesis in LTBMC.[7] Using a method similar to that described by Heard and colleagues, they demonstrated a dose-dependent increase in CSF when LTBMCs were exposed to ionizing irradiation. The CSF increase correlated inversely with the reduction in myelopoiesis induced by irradiation.

Recently, we developed a micromethod of LTBMC that allows initiation of numerous individual cultures from a single donor animal and makes possible frequent assessment of culture characteristics.[8] Using this method, we showed that a predictable increase in myelopoiesis occurs following weekly medium change. In this study, we examined the kinetics of day-to-day variations in myeloid cell production in vitro and the potential role of CSF in these events.

MATERIALS AND METHODS

Long-term bone marrow cultures were studied using a micromethod reported recently.[8] This is a modification of the method developed initially by Dexter and colleagues[1] and subsequently modified by Mauch and co-workers.[2] Marrow from the femora and tibia of 3-month-old C57BL/6 mice were flushed into α-medium (Flow Laboratories, McClean, VA) with a 1-mL syringe fitted with a 23-gauge needle. The cells were aspirated repeatedly to form a single cell suspension. After determination of the cell count with a model ZF Coulter counter (Coulter Electronics, Hialeah, FL), 1.4×10^6 cells were added to Linbro wells (Flow Laboratories) in a medium containing 65% α-medium, 25% horse serum, and 10^{-5} mol/L of hydrocortisone (10%) to a final volume of 0.7 mL. In some

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studies, cultures were set up identically except that hydrocortisone was omitted. The Linbro wells were placed in sterile covered Petri dishes and incubated in a water-jacketed incubator at 33°C in a humidified atmosphere with 5% CO_2. At weekly intervals, half the supernatant was removed from each well and replaced with an equal volume of fresh medium that was identical to that removed.

Colony-stimulating factor was measured serially in the culture supernatant starting 4 weeks after initiation of the cultures. In addition, the total number of cells in the culture and the fraction of the total that were either adherent layer cells or myeloid cells were determined. The culture supernatant was removed gently and centrifuged at 250 g for 10 minutes at 4°C and used for measurement of CSF as described below. α-Medium (0.5 mL) was added to the LTBMCs, and the adherent layers were freed using a rubber policeman. A single cell suspension was made by passage back and forth three times through a 23-gauge needle. These cells were mixed with those recovered from the supernatant and made up to a 1-mL vol. The cell count was determined in a Coulter counter and recorded as total cells per well. A cytospin preparation made from each cell suspension was stained with Wright's Giemsa, and a 200-cell differential count was performed on each. Cells were categorized as proliferative (myeloblast, promyelocyte, myelocyte) and nonproliferative (metamyelocyte, band, and polymorphonuclear neutrophil) myeloid cells and others (which included stromal cells).

Biologically active CSF was measured by assessing colony number when 1×10^5 fresh murine bone marrow cells were cultured for CFU-C in the presence of 10% supernatant from LTBMCs. The method used was that described by Worton and colleagues.[9] Medium consisted of 0.8% methylcellulose, 30% fetal bovine serum (FBS) (Flow Laboratories) and 1% bovine serum albumin (BSA) (Sigma, St Louis). The culture was added to a 35-mm dish and incubated for seven days in a water-jacketed incubator at 37°C in an atmosphere 5% CO_2. As a positive control, CFU-C were measured using LCM as the source of CSF.[10] The concentration of LCM used was that which caused maximal stimulation of colony formation. Additional cultures used, as a negative control, fresh medium used to refeed LTBMC. Three CFU-C cultures were set up for the assay of each sample. With an inverted microscope, colonies of $\geq$50 cells were counted on day 7 after the culture was initiated. Immunoreactive CSF was measured by a double-antibody radioimmunoassay (RIA).[11,12]

In some studies, the effect of adding small volumes of LCM, a tenfold concentrate of LTBMC supernatant obtained 24 hours after refeeding, or purified L cell CSF on myeloid cell production was examined. The L cell CSF was produced by culture of murine L cells in serum-free CMRL 1066 medium. A 10-L pool of conditioned medium was concentrated 250-fold by ultrafiltration using an Amicon PM 10 membrane. This material was applied to an affinity column to which purified anti-CSF was bound covalently. After being washed with buffer, the purified CSF was eluted with 2 mol/L of guanidine, pH 4.0[13] The final material appeared homogenous in sodium dodecyl sulfate (SDS)-acrylamide gel and had a specific activity of 7.5×10^7 U/mg of protein.

Antibodies to CSF were produced in a New Zealand white rabbit by multiple intradermal injections of 20 µg of purified CSF emulsified in Freund's complete adjuvant.[13-15] The antiserum used in these studies had a 50% neutralization titer of 1:2048 as judged by assay against an L cell CSF standard.

RESULTS

In a first series of experiments, LTBMC cell number, myeloid cells, and CSF from week-4 cultures were measured for 7 consecutive days beginning at the time of refeeding. Each day, six culture wells were examined; the results represent the mean (± SEM) of seven experiments conducted over 1 week (Fig 1). Refeeding of the culture increased the total cell number significantly three days later ($P < .001$), returning to the premedium change value by day 7. This cell increase was due exclusively to increases in the number of myeloid cells. Proliferative myeloid cells reached a peak two days after feeding, and nonproliferative myeloid cells achieved maximal levels four days after feeding (Fig 1B and C). The biologic CSF activity in the supernatant one day after refeeding was significantly higher than the value prior to refeeding ($P < .001$). CSF levels decreased over subsequent days, returning to basal levels by day 4 (Fig 1D).

To determine whether the increase in biologic CSF was paralleled by quantitative increases in immunologically measurable CSF, a study was undertaken in which LTBMC supernatant, obtained at daily intervals for 1 week, was simultaneously assayed for CSF by both bioassay and radioimmunoassay. The results showed a prompt rise 24

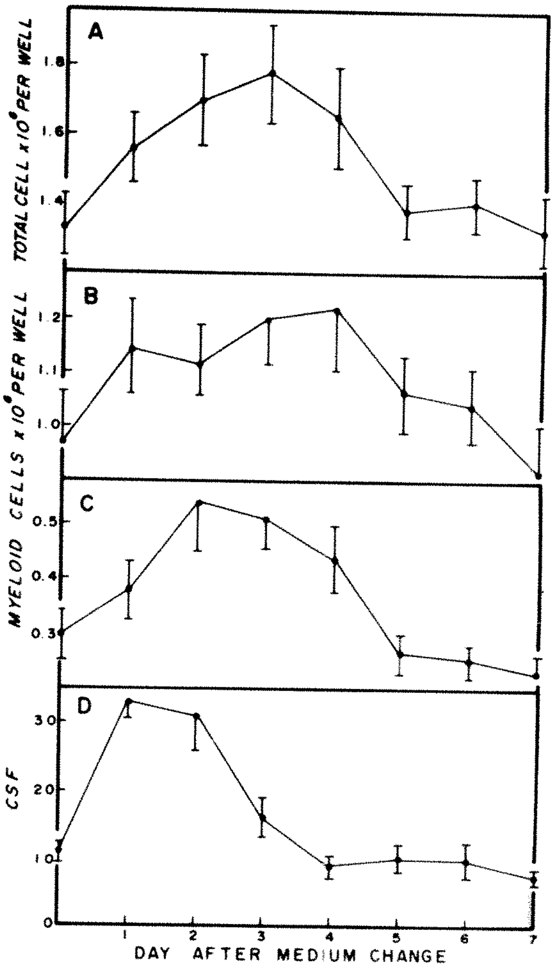

Fig 1. Daily fluctuations in total cell number (A) and in nonproliferative (B) and proliferative (C) myeloid cells in long-term bone marrow culture (LTBMC); biologic colony-stimulating factor (CSF) expressed as CFU-C/10^6 marrow cells (D). Results show daily measurements obtained from six wells prior to and for seven days after medium changes starting 4 weeks after culture initiation. Each value represents the mean of seven separate 1-week studies.

hours after feeding in both biologically active and immunologic CSF (Fig 2). Thereafter, both measurements returned toward basal levels.

In these studies, highly significant increases in CSF occurred prior to a wave of increased granulopoiesis. Previous reports showed that midweek feeding of LTBMCs increased total cell production.[16] We therefore investigated the effect of a midweek medium change four days after feeding and demonstrated a significant increase in total LTBMC cell and myeloid counts three days later (Fig 3). This wave of increased myelopoiesis was preceded by significant increases in biologic CSF that was maximal one day after midweek feeding.

Subsequent studies examined the effect on cell production of adding CSF alone to LTBMCs. In these experiments, three separate CSF preparations were studied. These included the addition of known concentrations of CSF contained in LCM, purified CSF prepared from L cell medium, or CSF contained in tenfold-concentrated supernatant obtained from LTBMCs 24 hours after medium change. In these studies, the refeeding of the fifth week (corresponding to day 7 of the studies described above) was replaced by the addition of 40 μL of the solution containing the CSF being tested. Control cultures received 40 μL of an identical solution that did not contain CSF. In these cultures, significant reductions in total cell numbers per well occurred over the subsequent days (Fig 4). In contrast, when 40 μL of LCM was added to cultures, a significant increase in total cell and myeloid number in each well occurred over the next three days ($P < .001$, Fig 4). A significant increase in total cells and myeloid cells in each well occurred when LCM containing similar levels of CSF was added to LTBMCs four days after refeeding. The effect of adding purified L cell CSF or CSF concentrated from LTBMC supernatant to the cultures is shown in Table 1. With either form of CSF, a significant increase in both total and myeloid cell production occurred ($P < .001$ for L cell:CSF, $P < 0.02$ for concentrated CSF).

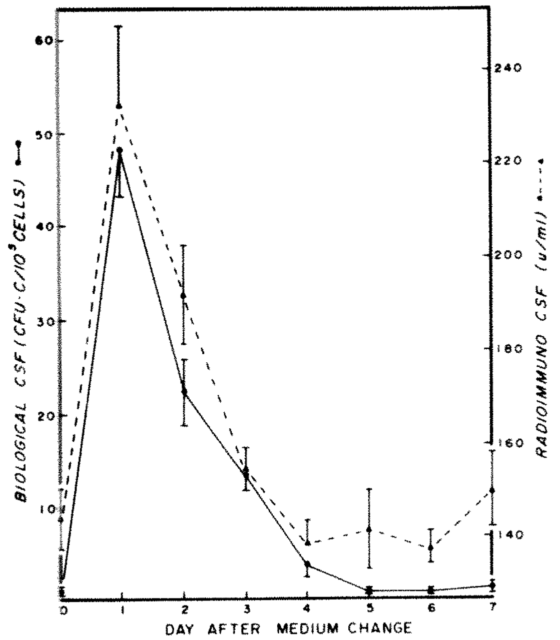

Fig 2. Comparison of biologically measurable colony-stimulating factor (CSF) (●—●) and immunoreactive CSF (▲---▲) in the supernatants of long-term bone marrow cultures (LTBMCs) prior to and for seven days after medium change. Study began 4 weeks after culture initiation. Each value is the mean of CSF levels in six cultures harvested simultaneously for biologic and immunologic assay.

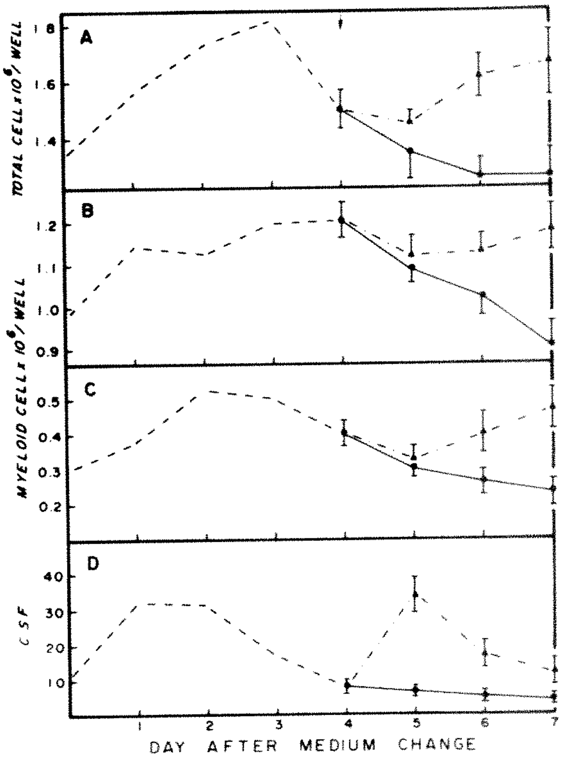

Fig 3. Effect of weekly medium change or midweek feeding on total cell number (A) and on nonproliferative (B) and proliferative (C) myeloid cells in long-term bone marrow cultures (LTMBCs); biologically measurable colony-stimulating factor (CSF) (expressed as CFU-C/10^5 marrow cells) (D). Dotted line for the first four days corresponds to the mean values shown in Fig 1 and is included to allow better interpretation of data. The effect of midweek feeding (▲—·—▲) with corresponding controls (●—●) was undertaken on day 4 and followed until day 7.

In LTBMCs with active hematopoiesis, fluctuations in CSF levels and myeloid cell counts occur when the culture medium is changed. A previous study by Heard and colleagues suggested that the fall in measurable CSF is related to consumption of this material by proliferative myeloid cells.[6] Hence, daily fluctuations of CSF were studied in a LTBMC free of myelopoiesis by examining cultures initiated in the absence of hydrocortisone. Four weeks after initiation, these cultures consisted exclusively of stromal-related cells. In these cultures, minimal fluctuations occurred in daily total cell number or CSF levels (Fig 5). The level of biologically active CSF at the time of medium change in these cultures was significantly higher than in hydrocortisone-containing cultures with active myelopoiesis ($P < .001$).

Following a peak at 24 hours after medium change, CSF

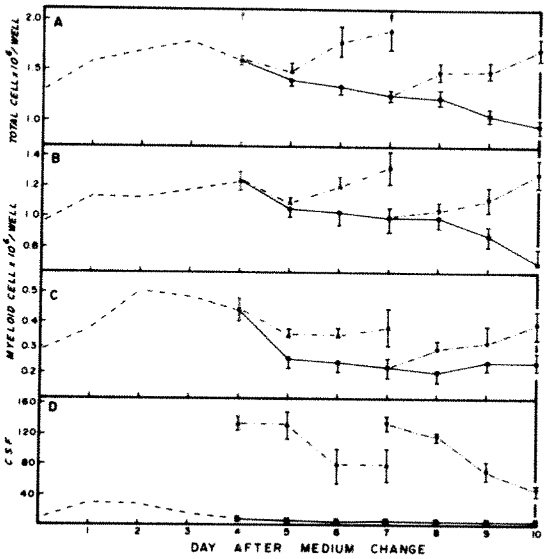

Fig 4. The effect of the addition of 40 μL of lung-conditioned medium (LCM) (containing a colony-stimulating factor (CSF) level of 136.6 ± 10.8 CFU-C/10^5 cells) to long-term bone marrow culture (LTBMC) four days or seven days after medium change. Dotted line for the first four days corresponds to the mean values shown in Fig 1 and is included to allow better interpretation of the data. The addition of LCM on day 4 (▲—·—▲) or day 7 (▼—·—▼) after medium change resulted in significant increases in total cell number (A), and in nonproliferative (B) and proliferative (C) myeloid cells. This increase is contrasted with reduction in cell numbers when control cultures received 40 μL of an identical solution not containing CSF on day 4 or day 7 (●——●). Biologic measurements of CSF (expressed as CFU-C/10^5 marrow cells) were increased in the supernatant on the day that LCM was added to the culture (D).

levels in LTBMCs markedly decreased over the subsequent days. The measured decrease in the biologic CSF level may be due to the presence of an inhibitor of CFU-C growth in the culture. To test this possibility, a biologic CSF assay was made in which the LTBMC supernatant, with an optimal concentration of LCM or LCM added that resulted in a 50% stimulation of CFU-C production, was tested for CFU-C growth. As shown in Fig 6, no inhibitor or promoter of CFU-C growth could be detected in this system.

The effect of adding an antiserum against L cell CSF to LTBMC at the time of medium change was then examined. Preliminary studies showed that a dilution of 1:100 of this antiserum neutralized 100% of the CSF contained in LTBCM supernatant 24 hours after medium change. Studies were made at the time of routine feeding 4 weeks after culture initiation. Antiserum, at a final dilution of 1:100, was added to half the cultures. The remaining cultures received an identical concentration of a control rabbit serum. The presence of antiserum in the medium totally neutralized supernatant CSF levels (Table 2). Despite a decrease in measurable CSF in the supernatant, no inhibition of cell production occurred in LTBMC. In a further study, the effect of adding a higher concentration of antiserum (1:17) to cultures was examined. Even at this level, no decrease in myelopoiesis occurred in cultures despite complete neutralization of supernatant CSF (Table 2).

DISCUSSION

Although CSF is essential for the clonal growth of committed myeloid progenitor cells in vitro, its role in the regulation of myelopoiesis in vitro and in vivo is not completely understood. The presence of CSF is required continually for primitive myeloid progenitor cells to mature into recognizable colonies.[17] Although correlative studies have suggested a relationship between levels of serum CSF and myelopoiesis,[18-20] speculation continues over the in vivo role of CSF. Recent evidence in LTBMC studies provides support for a regulatory role of CSF in myeloid cell proliferation. The results we describe complement the reports of Heard and colleagues and Gualtieri and co-workers, which demonstrated high CSF levels when myelopoiesis was decreased or suppressed and vice versa.[6,7] The method we used allowed examination of the relationship of CSF to myeloid cell production on a day-to-day basis. We demonstrated that weekly or midweek feeding of cultures was followed by a wave of increased myelopoiesis that was invariably preceded

Table 1. Effect of Addition of CSF on Total Cell Production in LTBMC

Days After Refeeding	Control		CSF in Lung-Conditioned Medium		CSF Concentrated From LTBMC Supernatant		Purified L Cell CSF	
	Cell Count*	CSF†	Cell Count	CSF	Cell Count	CSF	Cell Count	CSF
7	1.518‡	6.9	—	136.6	—	157.3	—	152.7
	(0.152)	(1.7)		(10.8)		(15.8)		(12.5)
8	1.415	4.0	1.510	118.0	1.564	85.6	1.725	151.6
	(0.115)	(1.5)	(0.186)	(3.7)	(0.195)	(5.7)	(0.169)	(8.6)
9	1.215	3.4	1.627	69.3	1.457	84.4	1.849	78.3
	(0.085)	(1.1)	(0.063)	(6.0)	(0.100)	(6.5)	(0.242)	(9.3)
10	1.108	3.8	1.703	49.8	1.641	17.0	1.814	55.6
	(0.069)	(1.1)	(0.143)	(7.6)	(0.043)	(1.8)	(0.075)	(3.5)

CSF, colony-stimulating factor; LTBMC, long-term bone marrow culture.

Forty microliters of CSF in LCM, purified L cell CSF, or CSF concentrated from LTBMC was added to culture seven days after medium change. Control cultures received 40 μL of an identical solution not containing CSF. The CSF concentration in LCM was 341.5 CFU-C/10^5 cells/mL. CSF concentration for L cell CSF was 381.7, and the concentrated supernatant from LTBMC was 393.5 CFU-C/10^5 cells/mL.

*Cell count × 10^6/well.

†CSF expressed as CFU-C/10^5 bone marrow cells.

‡Mean (± SEM).

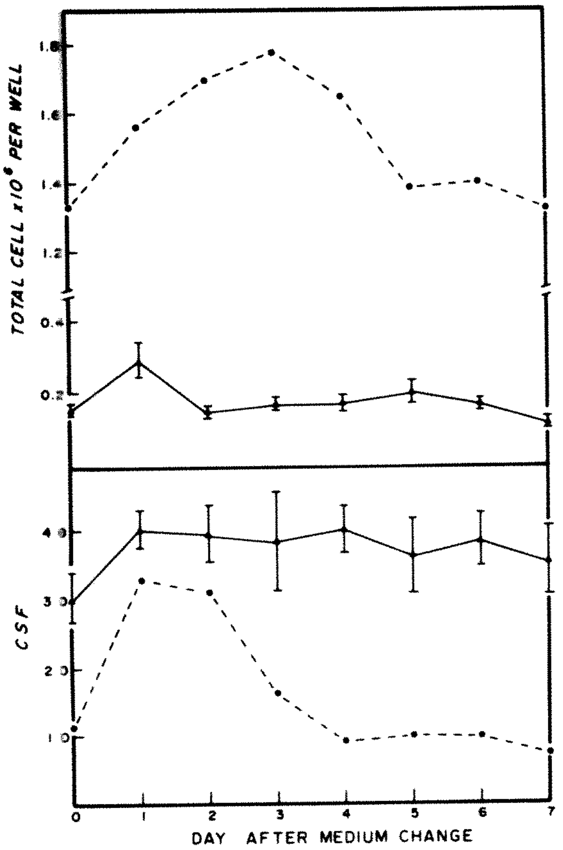

Fig 5. Comparison of daily total cell number (top) and biologic colony-stimulating factor (CSF) (bottom) in long-term bone marrow cultures (LTBMCs) initiated with (●---●) or without hydrocortisone (▲—▲). CSF is expressed as CFU-C/10^5 marrow cells.

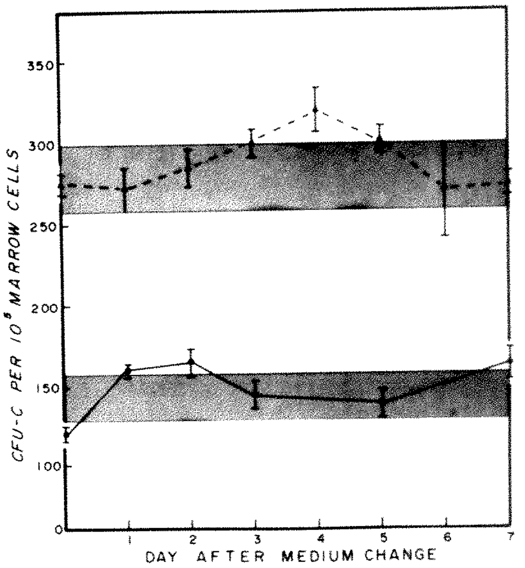

Fig 6. The presence or absence of promoters or inhibitors of colony-stimulating factor (CSF) in supernatant collected from long-term bone marrow cultures (LTBMCs) in Linbro-well cultures prior to and daily for seven days after medium change. Results demonstrated CFU-C production when LTBMC supernatant was cultured with lung-conditioned medium (LCM) that maximally stimulated CFU-C growth (▲---▲) or with LCM that stimulated CFU-C growth to 50% of maximum (●—●). Horizontal bars: mean (± SEM) CFU-C obtained when marrow was cultured for CFU-C with LCM alone.

by significant increases in CSF measured either biologically or immunologically, which strongly suggests a direct cause-and-effect relationship. This finding confirms a recent report showing an increase in CSF following medium change in flask cultures.[21] We believe that the failure to detect significant levels of CSF in culture in early studies is related to the time at which the supernatant was sampled (seven days after medium change).

We also, for the first time, demonstrated that the addition to LTBMCs of high concentrations of various forms of CSF alone (ie, without medium change) caused an increase in myeloid cell production that equaled that which occurred when whole medium was added to the culture. Similar increases in myelopoiesis were observed when the CSF contained in LCM, purified L cell CSF, or CSF concentrated from LTBMCs was added to the culture. This result contrasts to the study of Dexter and Shadduck,[4] who could not demonstrate an effect on myeloid cell production due to the addition of standard or pure L cell CSF to LTBMC. We believe that this is related to the dose of CSF added to culture (120 U/mL in the earlier study as compared with 380 U/mL in this report) and to examination of the culture only seven days after the addition of CSF. If these cultures had been examined daily, the results probably would have been similar to ours. The fact that L cell CSF stimulated myelopoiesis is of additional interest. This finding is not compatible with the belief that L cell CSF stimulates only macrophage proliferation. L cell CSF may also enhance myelopoiesis indirectly by stimulating microenvironmental cells to produce a myeloid cell-specific CSF.

CSF levels were increased in supernatants from LTBMC initiated in the absence of hydrocortisone. These cultures had an established adherent layer but did not produce myeloid precursors. The findings confirm previous reports that CSF levels increase in cultures in response to a reduction of myelopoiesis.[6,7] Consumption and degradation of CSF by proliferating myeloid cells has been reported previously and probably accounts for the lack of day-to-day fluctuations in CSF levels following medium change in these cultures.

Previous studies either did[22] or did not[6] show inhibitors to CSF in the supernatants of LTBMCs. Other researchers demonstrated colony-promoting activity (CPA), which enhances CFU-C production by recruitment of stem cells from a pre-CFU-C compartment.[23] To determine whether either inhibitors or promoters were present in the supernatant of LTBMCs on a day-to-day basis, we measured biologically active CSF in LTBMC supernatants mixed with LCM. This medium contained CSF that, either maximally or at 50% of the maximum, stimulated CFU-C growth in vitro. With this approach, no inhibitor or promoter could be detected. The experimental conditions that we used to seek promoters or inhibitors to myelopoiesis in culture were identical to those used in the previous study.[22] We have no explanation for the discrepancy between our results and the previous report.

Table 2. Effect of CSF Antiserum on Cell Production in LTBMC

	Medium Change Alone		Medium Change Plus Control Rabbit Serum (1:100)		Medium Change Plus Anti-CSF-Antiserum (1:100)		Medium Change Plus Anti-CSF-Antiserum (1:17)	
Days	Cell Count*	CSF†	Cell Count	CSF	Cell Count	CSF	Cell Count	CSF
0	1.325‡	1.0	—	—	—	—	—	—
	(0.075)	(0.5)						
1	1.358	30.9	1.475	32.9	1.570	0.8	1.425	0
	(0.158)	(5.4)	(0.134)	(5.5)	(0.080)	(0.4)	(0.093)	
2	1.797	25.3	1.706	15.0	1.670	0.7	1.825	0
	(0.332)	(2.3)	(0.142)	(5.6)	(0.259)	(0.5)	(0.085)	
3	2.086	10.2	2.100	3.0	1.929	0	2.177	0
	(0.208)	(4.3)	(0.252)	(1.7)	(0.269)		(0.123)	

Abbreviations as in Table 1.

Antiserum was added at the time of medium change (day 0). Control cultures received either refeeding medium alone or medium containing control rabbit serum.

*Cell count × 10^6 cells/well.

†CSF expressed as CFU-C/10^5 bone marrow cells.

‡Mean (± SEM).

Cells in the hematopoietic microenvironment evidently play an important role in the regulation of in vitro and in vivo myelopoiesis.[24] These cells, which include monocytes, macrophages, and fibroblastoid cells synthesize CSF, which in turn stimulates granulopoietic cells to divide. This function is believed to occur locally and to involve a CSF-mediated direct interaction between microenvironmental and myeloid cells. Our results, which demonstrated fluctuation in supernatant CSF that was temporally related to myelopoiesis, raise the possibility that CSF produced by the microenvironment is first excreted into the surrounding medium and then stimulates myeloid precursors. To test this possibility, we performed studies in which anti-CSF serum was added to LTBMC. Anti-CSF neutralized supernatant CSF, yet had no effect on myelopoietic cell production. This result supports the previous observation that the addition of anti-CSF to culture at the time of medium change had no effect on supernatant cell recovery.[4] A likely explanation of our results is that CSF levels in the supernatant must merely reflect CSF function within the microenvironment. This observation supports the concept that CSF acts by a cell–cell interaction between microenvironment and hematopoietic cells. Presumably, CSF in the medium is proportional to the CSF produced. Because CSF production precedes a wave of myelopoiesis, a role for the hormone in myelopoietic regulation is strongly supported. Because exogenous CSF also stimulates myelopoiesis, it too probably can react with microenvironmental cells to stimulate primitive myelopoietic cells to divide. Alternatively, the added CSF may stimulate microenvironmental cells to synthesize endogenous CSF or other factors, which in turn stimulates myelopoiesis. Because increased myelopoiesis occurs when CSF alone (ie, without medium change) is added to cultures, these results also indicate that CSF production rather than nutrient requirement limits the rate of granulopoiesis in LTBMC.

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Retinoic Acid Cooperates With Tumor Necrosis Factor and Immune Interferon in Inducing Differentiation and Growth Inhibition of the Human Promyelocytic Leukemic Cell Line HL-60

By Giorgio Trinchieri, Mark Rosen, and Bice Perussia

In this study, we analyzed the effect of tumor necrosis factor (TNF) on retinoic acid (RA)-induced myeloid differentiation of the promyelocytic HL-60 leukemia cell line. We show that low concentrations of the two substances, almost inactive in inducing differentiation when used separately, induce differentiation when added simultaneously to the cell cultures. Cells simultaneously expressing both monocyte/macrophage phenotype (typically induced by TNF) and granulocyte characteristics (typically induced by RA) are induced by a combination of the two factors, indicating that TNF and RA potentiate each other's activity. The results obtained using immune interferon (IFN-γ) in combination with the two inducers suggest that the mechanism of action of TNF and IFN-γ are possibly different. The inhibitory effect of RA on the expression of HLA class I antigens and of the high-affinity Fc receptor is potentiated by TNF but completely reversed by rIFN-γ.
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LEUKEMIA-DERIVED human myeloid cell lines, such as the promyelocytic HL-60,[1] are often used as models to study terminal differentiation of myelomonocytic cells. Dimethylsulfoxide (DMSO),[2] retinoic acid (RA),[3] and other substances induce HL-60 cells to differentiate along the myeloid lineage, whereas 12-*O*-tetra-decanoyl phorbol-13-acetate (TPA),[4] 1,25-dihydroxyvitamin D_3,[5] and leukocyte products contained in medium conditioned from phytohemagglutinin (PHA)-activated leukocytes (PHA-CM)[6-8] induce them to differentiate into cells with characteristics of monocyte/macrophages. We[9] demonstrated that PHA-CM in which immune interferon (IFN-γ) is neutralized with specific antibodies or antibodies from which IFN-γ has been removed by immune affinity chromatography is less potent than untreated PHA-CM in inducing differentiation of human myeloid cell lines and fresh myeloid leukemia cells. IFN-γ, however, exists in PHA-CM in concentrations insufficient to account for its differentiation-inducing activity, and other factors have been detected in IFN-γ–depleted PHA-CM that synergize with IFN-γ to induce differentiation.[9]

Recently, purification and cloning of the genes for tumor necrosis factor (TNF) and lymphotoxin (LT) have allowed more detailed studies of the biology of these cytotoxins.[10-14] The two cytotoxins also appear to mediate regulatory effects on various cell types, eg, they act as growth factors on fibroblasts[15,16] and regulate proliferation, differentiation, and functions of myelomonocytic cells at all stages of differentiation.[17] In particular, we demonstrated that a soluble factor that inhibits myeloid and erythroid colony formation in vitro is produced by natural killer (NK) cells[18] and has characteristics of TNF, and that LT and IFN-γ, produced by mitogen-activated T cells, act synergistically to block colony formation.[19] We also showed that cytotoxins TNF or LT at concentrations on the order of 10^{-11} mol/L induce monocytic differentiation of human myeloid cell lines[20] and act synergistically with IFN-γ.

The possibility of using inducers of differentiation for the therapy of leukemias or other tumors has recently received considerable interest. Of the many agents that induce differentiation of HL-60 cells, RA has until now been considered one of the inducers of possible therapeutic use because it is active in vitro at physiologic concentrations,[3] induces differentiation of fresh leukemia cells in primary cultures,[21] and is effective in vivo on patients with acute promyelocytic leukemia.[22,23] Cooperation between suboptimal concentrations of RA and the differentiation inducer(s) contained in PHA-CM in inducing differentiation of HL-60 cells have been reported.[24,25] LT[19] and low levels of TNF (G. Trinchieri, M. Rosen, and B. Perussia, unpublished observations) are contained in PHA-CM and, at least in part, are responsible for its differentiation-inducing activity, synergistic with IFN-γ, present in the PHA-CM. In the present study, we analyzed the combined effect of TNF and RA on the differentiation of HL-60 cells. Concentrations of the two substances, almost inactive by themselves, induced most HL-60 cells to differentiate when added simultaneously to the cultures. The combined effect of the two substances powerfully induced cells simultaneously expressing both monocyte/macrophage markers, typically induced by TNF, and granulocyte characteristics, typically induced by RA.

MATERIALS AND METHODS

Monoclonal antibodies. Monoclonal antibodies B9.8, B52.1, B33.1, and B137.17 were produced and characterized in our laboratory; OKM1, 5E9, W6.32, and BBM1 were produced from cell lines obtained from the American Tissue Culture Collection (Rockville, MD); 3G8 was kindly donated by Dr J. Unkeless (Mount Sinai School of Medicine, New York), and KuFc79 was a gift of Dr P. Gambel (Medical College of Virginia). Antibodies B9.8 (IgM,[26]) and OKM1 (IgG2b, anti-C3bi receptor,[27]) react with two distinct antigens expressed on all differentiated neutrophilic granulocytes and monocytes and start to be expressed, during differentiation, at the myelocytic and promyelocytic stages, respectively. Antibody B52.1 (IgM,[8]) reacts with all peripheral blood monocytes and all

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α-naphthyl acetate esterase (ANAE)-positive cells in the bone marrow, and cross-competes for binding to monocytes with antibody Mo2.[28] Antibody W6.32 (IgG2a,[29]) reacts with HLA class I antigens, antibody BBM1 (IgG2a,[30]) reacts with β_2-microglobulin, and antibody B33.1 (IgG2a,[31]) reacts with HLA class II antigens. Antibody 5E9 (IgG1,[32]) reacts with the transferrin receptor. Antibody 3G8 (IgG1,[33]) reacts with a surface molecule of mol wt 50,000 to 72,000 identified as the low-affinity receptor for aggregated IgG on neutrophilic granulocytes, NK cells, and macrophages. The mouse monoclonal IgG2a B137.17 is an Ig of unknown specificity selected on the basis of its high-affinity binding, in monomeric form, to the IFN-γ–inducible FcR on monocyte and myeloid cell lines.[34] The binding of B137.17 to cells is Fc fragment dependent, and is blocked by monomeric human IgG1 and IgG3 and murine IgG2a and IgG3. Sepharose-linked B137.17 precipitates from human monocytes and myeloid cell lines a molecule of mol wt 70,000, identified as the high-affinity FcR for monomeric IgG. Antibody KuFc79 (IgG2b,[35]) reacts with a 42,000-dalton FcR species present on monocytes, granulocytes, and B cells.

Cytokine preparations. Purified human recombinant IFN-γ (rIFN-γ) from *Escherichia coli* was kindly supplied by Dr H. M. Shepard (Genentech, South San Francisco) and has an antiviral activity titer of 7×10^7 U/mg of HeLa cells. Two preparations of human recombinant TNF (rTNF) were used in this study with identical results: one (90% pure, 10^7 U/mg as tested on L-929, α subline, cells) was kindly supplied by Dr J. S. Price (Cetus, Emeryville, CA), and the other (electrophoretically homogeneous, 5×10^7 U/mg) was supplied by Dr H. M. Shepard (Genentech).

Induction of myeloid cell lines. HL-60 cells were seeded at 1.5×10^5 cells/mL in RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 15% fetal bovine serum (FBS). The various inducers of differentiation were present throughout the culture period. All-*trans*-RA (Sigma) was dissolved in ethanol (0.3 mg/mL).

Cytochemistry. Staining for ANAE was according to Platt.[36] Ability of the cells to reduce nitroblue tetrazolium (NBT, Sigma) was determined by mixing on a microscope slide 1 vol of cells in medium (5×10^6 cells/mL) and 1 vol of 1 mg/mL of NBT in NaCl 0.15 mol/L. After a 30-minute incubation at 37°C in a humidified atmosphere, slides were dried, stained with Wright's-Giemsa, and the percentage of cells containing blue-black formazan deposits was scored from at least 200 cells.

Indirect immunofluorescence, cell cycle analysis, and ^{3}H-thymidine (^{3}H-TdR) incorporation. Indirect immunofluorescence (flow cytometry), cell cycle analysis by staining of DNA using propidium iodide, and ^{3}H-TdR incorporation, were performed as previously described in detail.[20]

RESULTS

Morphological changes in HL-60 cells cultured with RA and TNF. When cultured in 300 nmol/L of RA for five days, most HL-60 cells acquired the morphology of mature myeloid cells, from myelocytes to polymorphonuclear cells (Fig 1C). Little or no morphological differentiation was observed with 10 nmol/L of RA (Fig 1B). Treatment of HL-60 cells with rTNF (10 U/mL) resulted in minimal morphological changes: TNF-treated HL-60 cells (Fig 1D) became smaller and more irregular, and the proportion of cells with nucleoli and primary granules was lower than in control untreated cultures. In cultures with 10 nmol/L of RA and 10 U/mL of rTNF, with or without rIFN-γ, a large proportion of HL-60 cells appeared to have mature myeloid morphology, including polymorphonucleated cells (Fig 1E). Addition of 10 U/mL of rTNF to HL-60 cell cultures in the presence of 300 nmol/L enhanced the formation of cells with mature myeloid morphology: Almost all HL-60 cells on day 5 of culture acquired the morphology of polymorphonuclear granulocytes (Fig 1F).

Induction of differentiation of HL-60 cells by TNF and RA. As previously shown,[20] a proportion of HL-60 cells cultured for five days in the presence of TNF expressed the monocyte-specific antigen recognized by antibody B52.1, the myelomonocytic antigen OKM1 at higher density than untreated cells, ANAE activity, and ability to reduce NBT (Fig 2 and Table 1). The expression of the myelomonocytic antigen recognized by antibody B9.8 was also increased (not shown), whereas the proportion of cells bearing transferrin-

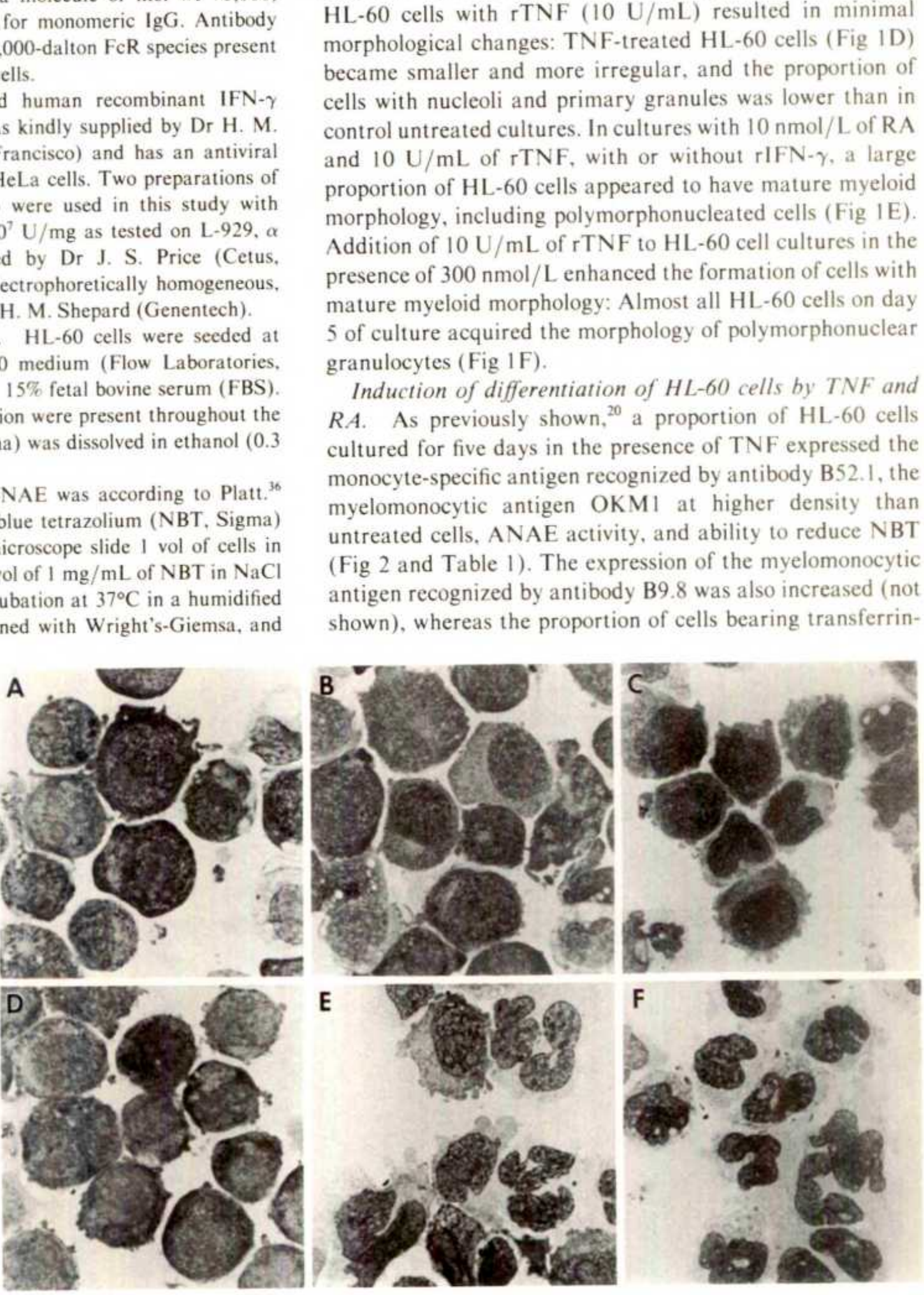

Fig 1. Morphology of HL-60 cells cultured in the presence of retinoic acid (RA) and/or recombinant tumor necrosis factor (rTNF). Cytocentrifuge smears were prepared from HL-60 cells, cultured for five days in the presence of the inducers, and stained with May-Grunwald-Giemsa. HL-60 cells were cultured in: (A) culture medium, or in culture medium supplemented with (B) 10 nmol/L of RA; (C) 300 nmol/L of RA; (D) 10 U/mL of rTNF; (E) 10 U/mL of rTNF and 10 nmol/L of RA; (F) 10 U/mL of rTNF and 300 nmol/L of RA. (Magnification ×690.)

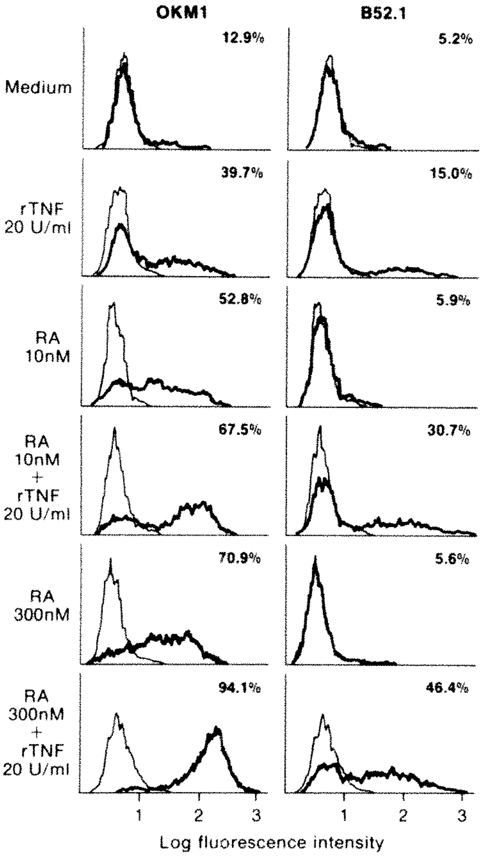

Fig 2. Expression of surface antigens on HL-60 cells cultured with retinoic acid (RA) and/or recombinant tumor necrosis factor (rTNF). The phenotype of HL-60 cells, cultured for five days with the addition of the indicated inducers, was studied using the indicated antibodies. On the histograms, the *x* axis represents the intensity of green fluorescence on an exponential scale; the *y* axis represents the number of cells; the thin line histograms represent the fluorescence of control cells in the absence of the monoclonal antibodies; the thick line histograms represent the fluorescence of the cells stained with the indicated monoclonal antibodies. The values in the top right corner of each histogram are the percentages of positive cells, calculated as described in the Materials and Methods section. Results are representative of six experiments with comparable results.

receptor, as detected using antibody 5E9, was slightly decreased (Fig 3). rIFN-γ alone (100 U/mL) had only a minimal effect on the expression of monocytic or myelomonocytic antigens and on ANAE or NBT reduction activities (Figs 2 and 3B, Table 1). Addition of rIFN-γ to TNF potentiated its ability to induce differentiation markers in HL-60 cells: previously reported experiments[20] demonstrated that the two factors act synergistically. Most HL-60 cells were induced by 300 nmol/L of RA to express the myelomonocytic antigens recognized by antibodies OKM1 (Fig 2) and B9.8 (not shown); no induction was observed using 10 nmol/L of RA; however, 10 nmol/L of RA potentiated the ability of rTNF, alone or in combination with rIFN-γ, to induce the two antigens. The number of HL-60 cells reducing NBT was modestly increased by 10 and 300 nmol/L of RA; RA, at both concentrations, acted synergistically with rTNF and rIFN-γ in inducing NBT reduction activity in

Table 1. Induction of ANAE Activity and NBT Reduction Activity in HL-60 Cells Cultured with rTNF, rIFN-γ, and RA

Treatment			ANAE Positive Cells Percent	NBT Reduction Positive Cells Percent
RA (nmol/L)	rIFN-γ (U/mL)	rTNF (U/mL)		
—	—	—	1.4	1.7
—	100	—	3.7	8.0
—	—	10	14.3	17.7
—	100	10	40.2	59.6
10	—	—	1.6	12.3
10	100	—	4.4	14.3
10	—	10	11.8	43.3
10	100	10	81.8	93.1
300	—	—	3.9	22.3
300	100	—	6.2	71.1
300	—	10	53.0	68.6
300	100	10	77.9	86.0

ANAE, α-naphthyl acetate esterase; rTNF, recombinant tumor necrosis factor; rIFN-γ, human recombinant interferon-γ, RA, retinoic acid; NBT, nitroblue tetrazolium.

HL-60 cells were cultured for five days with the indicated inducers and analyzed for ANAE and NBT reduction activities as described. Results are from one representative experiment of four experiments performed with similar results.

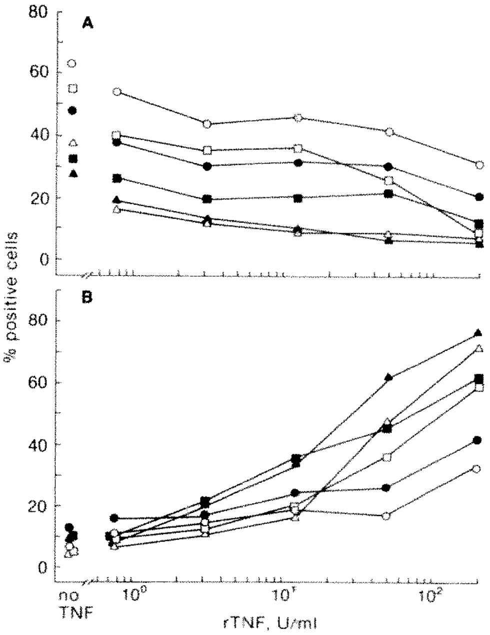

Fig 3. Synergistic effect of recombinant tumor necrosis factor (rTNF), human recombinant interferon-γ (rIFN-γ), and retinoic acid (RA) on the expression of the transferrin receptor, detected by antibody 5E9 (A), and of the monocyte-specific antigen detected by antibody B52.1 (B). HL-60 cells were cultured for five days with the indicated concentrations of rTNF in medium containing or not containing RA and rIFN-γ, washed, and tested by indirect immunofluorescence (flow cytometry) for expression of the indicated surface markers. HL-60 cells were cultured in medium containing: neither RA nor rIFN-γ (○); 100 U/mL of rIFN-γ (●); 10 nmol/L of RA (□); 10 nmol/L of RA and 100 U/mL of rIFN-γ (■) 300 nmol/L of RA (△); and 300 nmol/L of RA and 100 U/mL of rIFN-γ (▲).

HL-60 cells (Table 1). The expression of transferrin receptor as detected by antibody 5E9 (Fig 3A) was decreased by RA, and, more efficiently, by RA and rTNF; no synergism between rIFN-γ and RA in inhibiting transferrin receptor expression was observed. Finally, two monocytic markers, ie, the antigen recognized by antibody B52.1 (Fig 2) and ANAE activity (Table 1), were not induced by RA alone, but RA, at both 10 and 300 nmol/L, strongly potentiated the ability of rTNF, alone or in combination with rIFN-γ, to induce these markers in HL-60 cells. rTNF, at doses as low as 1 U/mL, significantly potentiated the effect of RA in reducing expression of transferrin receptor (Fig 3A), whereas the effect on the expression of B52.1 antigens was observed at rTNF doses $\geq$3 U/mL (Fig 3B). The effect on the expression of the two antigens was dose-dependent up to the highest concentration tested (200 U/mL of rTNF) and was potentiated by the presence of 100 U/mL of rIFN-γ (Fig 3). The analysis of these quantitative data demonstrated that the effects of the combination of RA and TNF on surface antigens expression are synergistic. A similar conclusion was derived from dose–response experiments (not shown) on the induction of ANAE activity and NBT reduction activity.

Cooperation between RA and rTNF in inhibiting proliferation of HL-60 cells. RA at 300 nmol/L almost completely suppressed ^{3}H-TdR incorporation by HL-60 cells cultured for five days (Table 2). At 10 nmol/L of RA, the inhibition was partial but became almost complete in the presence of rTNF. rIFN-γ did not potentiate the ability of RA to suppress ^{3}H-TdR incorporation in these cultures. Cell cycle analysis of proliferation revealed that almost no HL-60 cells were in S phase on day 5 of culture in the presence of 300 nmol/L of RA, rTNF, and rIFN-γ, but that a proportion of cells was in G2 phase after culture in the presence of 300 nmol/L of RA. At 10 nmol/L of RA, only a modest effect was observed on the distribution of the cells in the cell cycle, but addition of rTNF produced a profound decrease of cells in S phase, with some cells remaining in G2 phase, as was observed at higher RA concentrations.

Expression of different FcR types and HLA class I antigens on HL-60 cells induced with rTNF, rIFN-γ, and RA. The results of the analysis of FcR expression on HL-60 cells during differentiation are shown in Table 3. The low-affinity FcR of neutrophils, NK cells, and macrophages recognized by antibody 3G8 was poorly induced by rTNF, rIFN-γ, or RA alone, but was significantly induced by the combination of RA with rTNF or rIFN-γ. When HL-60 cells were cultured in the presence of the three inducers together, 3G8 antigen was induced in almost 50% of the cells. The expression of the 42,000-dalton FcR of neutrophils, monocytes, and B cells recognized by antibody KuFc79 was not significantly affected by rTNF and rIFN-γ, but the proportion of positive cells was strongly reduced in cultures with 10 or 300 nmol/L of RA. The inhibition of KuFc79 antigen expression by RA was partially reversed when either rTNF or rIFN-γ was present in the cultures, and the reversal was even greater when both were present. Expression of the high-affinity FcR for monomeric IgG, detected by binding of monoclonal IgG2a B137.17, was strongly enhanced by rIFN-γ but not by rTNF (Table 3). rTNF, however, potentiated the effect of rIFN-γ in inducing the receptor. RA, at 10 or 300 nmol/L, strongly suppressed the expression of the high-affinity FcR; rTNF potentiated the inhibitory effect of RA on induction of this FcR, whereas rIFN-γ completely reversed it.

Both rTNF and rIFN-γ increased expression of HLA class I antigens (as detected by antibody W6.32) and β_2-microglobulin (as detected by antibody BBM1) on HL-60 cells when used alone; a much better induction was observed in most but not all experiments when a combination of the two cytokines was used (not shown). RA at 300 but not at 10 nmol/L decreased expression of HLA class I antigens on HL-60 cells. rTNF, which by itself increases HLA class I antigen expression on HL-60 cells, potentiated the inhibitory effect of 300 nmol/L of RA and decreased HLA class I antigens expression in the presence of 10 nmol/L of RA. Unlike rTNF, rIFN-γ strongly increased HLA class I antigen expression in the presence of either 10 or 300 nmol/L of RA.

DISCUSSION

RA is a potent inducer of myeloid differentiation in vitro of promyelocytic cell lines and of fresh leukemic cells from patients with acute promyelocytic M3 leukemia.[34-36] The

Table 2. Effect of rTNF, rIFN-γ, and RA on the Proliferation of HL-60 Cells

Inducer	^{3}H-TdR Uptake cpm (% Inhibition)	Percentage of Cells in		
		G0/G1	S	G2/M
None	96,655	48.9	33.4	17.7
rIFN-γ, 100 U/mL	78,090 (20.2)	56.8	35.0	8.2
rTNF, 20 U/mL	46,572 (51.8)	55.6	33.2	11.1
rIFN-γ, 100 U/mL + rTNF, 100 U/mL	33,095 (65.8)	62.9	32.5	4.6
RA, 10 nmol/L	26,255 (72.8)	69.2	22.6	8.3
RA, 10 nmol/L + rIFN-γ, 100 U/mL	20,321 (79.0)	64.9	17.0	18.2
RA, 10 nmol/L + rTNF, 20 U/mL	3,462 (96.4)	83.0	6.0	10.8
RA, 10 nmol/L + rIFN-γ, 100 U/mL + rTNF, 20 U/mL	5,349 (94.5)	89.3	1.7	9.0
RA, 300 nmol/L	2,041 (97.9)	80.9	7.0	12.0

Abbreviations as in Table 1.

HL-60 cells were cultured for five days with the indicated inducers. To evaluate ^{3}H-TdR uptake, cells were cultured in 96-well flat-bottomed microtiter plates (initial concentration 2 × 10^4 cells/well in 200 μL). Cultures were pulsed with ^{3}H-TdR 1 μCi/well for six hours before harvesting. For cell cycle analysis (flow cytometry analysis of propidium iodide (PI)-stained cells), cells were cultured in flasks (initial concentration 1.5 × 10^5 cells/mL). Data are from one representative experiment of three experiments performed with similar results.

Table 3. Expression of FcR on HL-60 Cells Treated With rTNF, rIFN-γ, and RA

Monoclonal Antibody	Treatment[*]											
					RA 300 nmol/L				RA 10 nmol/L			
	None	rTNF	rIFN-γ	rTNF +rIFN-γ	—	rTNF	rIFN-γ	rTNF +rIFN-γ	—	rTNF	rIFN-γ	rTNF +rIFN-γ
None	1.0[†]	2.0	2.1	3.2	2.9	1.0	2.9	2.6	0.4	0.6	0.7	5.6
3G8	10.7	18.5	18.7	25.1	15.1	40.0	33.7	44.7	11.8	28.7	18.2	42.0
	(72)[‡]	(75)	(87)	(84)	(86)	(94)	(87)	(81)	(87)	(98)	(89)	(76)
KuFc79	83.2	78.2	72.4	77.7	31.5	55.0	51.5	64.4	30.9	45.3	31.1	58.2
	(43)	(44)	(45)	(51)	(67)	(63)	(63)	(82)	(58)	(64)	(56)	(71)
B137.17	83.9	71.2	93.4	94.5	49.1	11.8	89.7	89.0	48.6	37.0	94.2	86.2
	(53)	(44)	(104)	(133)	(33)	(13)	(120)	(85)	(30)	(24)	(117)	(82)

Abbreviations as in Table 1.

[*] HL-60 cells were cultured for five days with rTNF (20 U/mL), rIFN-γ (100 U/mL), RA (300 or 10 nmol/L), or a combination of the various factors. Results are representative of six experiments with comparable results.

[†] Percentage of positive cells as detected by indirect immunofluorescence (flow cytometry). The threshold fluorescence intensity at which 99% of the cells without first antibody added were negative was determined on HL-60 cells cultured in the absence of differentiation inducers and used for all the samples.

[‡] Numbers in parentheses indicate average intensity of fluorescence (on an arbitrary linear scale from 1 to 200) of positive cells.

human histiocytic cell line U937 is also induced to differentiate by RA,[37] but the human erythromyeloid leukemic cell line K562 and the myeloblastic leukemic cell line KG1[38] are not. RA, however, is a potent inhibitor of clonal growth not only of the HL-60 line, but also of the KG1 cell line that is not induced to differentiate by it,[38] and of leukemic cells freshly obtained from chronic myelogenous leukemia (CML) or from acute myelogenous leukemia (AML) patients[38] of M1, M2, and M3 stages. The concentrations of RA active in vitro in inducing differentiation of fresh leukemic cells or leukemia-derived cell lines as well as those inducing inhibition of clonal growth are in the order of 10^{-9} to 10^{-8} mol/L and are obtainable in vivo without prohibitive side effects.[22,23,39] In addition, RA is not toxic to normal hematopoietic precursor cells, but, on the contrary, significantly enhances the growth of normal hematopoietic colonies.[40,41] Other potent inducers of differentiation of myeloid cells such as TNF have, unlike RA, high systemic toxicity and are potent inhibitors of proliferation of both normal and leukemic hematopoietic progenitor cells, as well as of other cell types.[18] These activities limit their therapeutic potential for treatment of leukemias or other malignancies. The synergistic interaction of RA with other differentiation-inducing factors offer the promising possibility to induce differentiation and growth inhibition of leukemic cells at concentrations of either factor that might not affect normal precursor cells or have a generalized toxic effect.

RA is strongly synergistic with TNF in inducing differentiation of HL-60 cells, even at concentrations of 10^{-8} mol/L which, in the absence of TNF, are ineffective in inducing differentiation. The combination of 10^{8} mol/L of RA and 10^{-10} mol/L of TNF halts growth of HL-60 cells and induces expression of differentiation markers in almost all cells. An at least additive effect of IFN-γ and RA in inducing differentiation of U937 cells has been reported by Gullberg and colleagues.[42] The synergy of IFN-γ with RA is not as great as that of TNF and RA in inducing differentiation or growth inhibition of HL-60 cells, but the combination of the three factors often induces the most complete differentiation.

When TNF is added to HL-60 cells cultured with doses of RA able to induce myeloid differentiation (3×10^{-7} mol/L), the generation of mature myeloid cell types is not prevented and the induced cells, although expressing specific monocytic markers such as ANAE and B52.1 antigen, have typical mature myeloid morphology. When TNF is added with 10^{-8} mol/L of RA, a concentration unable to induce morphological maturation of HL-60 cells, mature myeloid cell types are induced in the cultures. Thus, not only does RA potentiate the monocytic differentiation induced by TNF, but TNF potentiates the myeloid differentiation induced by RA, and cells with intermediate myelomonocytic characteristics are generated. Analogously, B52.1 monocyte-specific antigen expression is induced on HL-60 cells during the myeloid differentiation induced by DMSO[8] but not by RA, and intermediate myelomonocytic characteristics are induced in a subset of chronic myelogenous leukemia cells by PHA-CM and IFN-γ.[7] This lineage ambiguity is not peculiar to leukemic cells, because similar results have been obtained with normal bone marrow immature myeloid cells when forced to differentiate in vitro.[7]

The interplay between TNF, IFN-γ, and RA in regulating expression of differentiation markers on HL-60 cells is best illustrated by the effect on HLA class I antigens and on the different types of Fc receptor. Both TNF and IFN-γ increase expression of HLA class I antigens, as previously described with other cell types.[43] The mechanism of action of the two cytokines is probably different, however, as suggested by results obtained in experiments in which HL-60 cells were tested with RA added. RA alone drastically decreases the expression of HLA class I antigens and TNF potentiates this inhibitory effect, whereas IFN-γ completely reverses it, inducing high expression of HLA class I antigens on most cells. The increased expression of these antigens during monocytic differentiation and their decreased expression during myeloid differentiation parallel the high and low expression of HLA class I antigens on mature monocyte and granulocytes, respectively. The pattern of high-affinity FcR induction on HL-60 cells by the differentiation agents is almost identical to that of HLA class I antigens. Again, the induction of high-affinity FcR during monocytic differentiation of HL-60 cells and its decreased expression during myeloid differentiation parallel the distribution of the FcR on normal differentiated cells, ie, high-level expression on monocytes and lack of expression on granulocytes, although in the latter cell type the high-affinity FcR can be induced by IFN-γ.[34,44] The low-affinity FcR present on neutrophils but not on monocytes, as detected by antibody 3G8,[33] is expressed at higher levels on HL-60 cells when the myeloid differentiation induced by RA is potentiated by TNF. Expression of a third type of FcR recognized by antibody KuFc79[35] during the differentiation of HL-60 cells is independent of that of both the high-affinity and low-affinity FcR detected with IgG2a B137.17 and with antibody 3G8, respectively.

The synergism of TNF with RA is similar to that reported by Olsson and colleagues for PHA-CM[25] and for the differentiation-inducing factor purified from the HUT-102 cell line.[45] LT and, in some preparations, TNF, are responsible, at least in part, for the differentiation-inducing activity, synergistic with RA, present in PHA-CM,[19] whereas the differentiation factor produced by HUT-102 cells seems to be distinct from both LT and TNF on the basis of its biochemical characteristics.[45] Understanding of the biochemical mechanism of the synergistic effect between TNF and RA might furnish important information on the regulation of growth and differentiation of normal and leukemic hematopoietic cells. To evaluate the therapeutic potential of the combined effect of the two inducers, it will be important to evaluate whether the combined substances affect normal hematopoietic precursor cells in vitro and whether leukemic cell types, not affected by RA or TNF alone, are induced to differentiate and lose their proliferative ability with the combined treatment.

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Pattern of Expression of HLA-DR and HLA-DQ Antigens and mRNA in Myeloid Differentiation

By Thomas T. Amatruda III, Roger Bohman, John Ranyard, and H. Phillip Koeffler

We examined the expression of HLA-DR and HLA-DQ antigens and mRNA from myeloid and lymphoid cells obtained from normal volunteers and established cell lines. Cytofluorometric analysis and immunoprecipitation were performed using murine monoclonal antibodies specific for HLA-DR (L-243) and HLA-DQ (Leu 10). The expression of mRNA for HLA-DR and HLA-DQ chains was determined by Northern blot and RNA dot-blot analysis. Lymphoid cell lines expressed both HLA-DR and HLA-DQ antigens, with consistently higher levels of expression of DR. Myeloid cell lines of early myeloblast or bipotent (myeloid-erythroid) phenotype (KG-1, KG-1a, HEL) expressed HLA-DR at high levels, whereas cell lines manifesting a greater degree of myeloid maturation (ML-3, HL-60, U937) expressed DR at low or undetectable levels. The HLA-DQ antigen was expressed at low levels on the surface of KG-1 and KG-1a cells but was not detectable on other myeloid cell lines. The expression of mRNA for HLA-DR and HLA-DQ chains paralleled the pattern of expression of the respective antigens. The HL-60 and U-937 cells stimulated to differentiate in vitro to macrophages with 1,25 dihydroxyvitamin D_3 [1, $25(OH)_2D_3$] were induced to express detectable levels of HLA-DR antigens. Exposure to γ-interferon (γ-IFN) increased the expression of HLA-DR antigens by all myeloid cell lines. Induction of differentiation in vitro with either $1,25(OH)_2D_3$ or dimethyl sulfoxide potentiated this effect of γ-IFN. Expression of the HLA-DQ antigens was increased on KG-1 myeloblasts after exposure to γ-IFN. HLA-DQ expression could not be detected on other myeloid cell lines after exposure to γ-IFN, nor was HLA-DQ expression stimulated by γ-IFN after HL-60 and U-937 cells were induced to differentiate to macrophagelike cells in vitro. These results provide additional evidence that expression of the HLA-DR and HLA-DQ genes may be independently regulated in human myeloid cells.

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ALTHOUGH EXPRESSION of MHC class I antigens is ubiquitous on murine and human cells, the pattern of expression of MHC class II antigens is tissue-specific and varies during differentiation. In humans, hematopoietic precursor cells express HLA-DR antigens but their mature progeny do not, with the exception of macrophages.[1-6] Expression of the other class II antigens DQ (DC, DS, MB) and SB has been less completely characterized. In previous studies on myeloid cells, expression of DQ antigens has apparently been at lower levels than DR expression.[7-9]

Expression of MHC class II antigens by monocytes and macrophages is of functional significance because these cells act as antigen-presenting cells (APCs) in generation of the immune response.[10-12] Antigen-presenting cells are characterized by their ability to act as accessory cells in stimulation of T cell proliferation in vitro and generation of antigen-specific T cell populations in vivo. These functions require the expression of appropriate MHC class II antigens, in addition to other properties specific to reticuloendothelial cells, such as phagocytosis and synthesis of degradative enzyme systems. The MHC type II antigens on the monocyte surface may play a role as a specific binding site for the T cell antigen receptor.[13] Neither immature myeloid cells nor cells from human myeloid cell lines can function as APCs, although cells from myeloid lines may acquire the ability to stimulate T cell proliferation after differentiation of these cells in vitro.[14]

We previously reported that cells of a human promyelocytic leukemia cell line, HL-60, are induced to express HLA-DR antigen by exposure to recombinant human γ-interferon γ-IFN.[15] These cells failed to express detectable levels of HLA-DQ (DC) antigen or mRNA. We and others demonstrated that ~50% of human peripheral blood monocytes are discordant in expression of HLA-DR and HLA-DQ (HLA-DR+, HLA-DQ−).[7,8,15-17] Exposure of monocytes to γ-IFN restored expression of HLA-DR and DQ to a variable extent. Taken together, these results suggest that the expression of HLA-DR may be independently regulated in human myeloid cells.

In the present study, we examined the pattern of expression of HLA-DR and HLA-DQ antigens and mRNA in lymphoid and myeloid cell lines at several stages of differentiation. In addition, we used the myeloid cell lines to study the ontogeny of MHC class II antigen expression induced by γ-IFN.

MATERIALS AND METHODS

RNA isolation, hybridization, and autoradiography. Hematopoietic cells were homogenized in guanidine thiocyanate (Eastman Kodak, Rochester, NY), and RNA from the homogenate was isolated by centrifugation through a cesium chloride cushion as previously described.[18] The poly (A)-containing RNA was enriched by oligo (dT)-cellulose chromatography.[19] Five micrograms of mRNA from each cell type were treated with 20 mmol/L of methyl mercuric hydroxide and electrophoresed in a 1.1% agarose/formaldehyde gel buffered with MOPS. Subsequently, the gel was photographed, equilibrated in 20 × SSC (standard saline citrate; 3 mol/L of NaCl, 0.3 mol/L of Na acetate, pH 6.8) for one hour with gentle rocking, transferred to Zetaprobe nylon membrane (Biorad, Richmond, CA) using 10 × SSC, prehybridized and hybridized to specific cDNA probes.[20,21] The cDNA probes were nick-translated[22]

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and used at a specific activity $>10^8$ cpm/μg. Hybridized filters were washed in 2 × SSC at room temperature, and in 2×, 1×, 0.5× and 0.1 × SSC containing 0.1% sodium dodecyl sulfate (SDS) at 68°C for 10 minutes each. The filters were exposed to x-ray film (Kodak XAR-5) for varying lengths of time at −70°C with an intensifying screen (DuPont, Wilmington, DE). Size of hybridizing RNA fragments was determined by comparison with 28S and 18S ribosomal RNA bands.[23] To reuse Zetaprobe blots, the first probe was eluted by washing the filter in 0.1 × SSC with 0.1% SDS at 95°C for two 20-minute washes. The filter was autoradiographed to document removal of probe, and a second hybridization was performed. The relative intensity of hybridization of the cDNA probes to RNA blots was quantitated by laser densitometry, using a Joyce-Loeble densitometer.

The cDNA clones used to probe RNA blots were described previously. The cDNA from tubulin[24] was generously provided by W. Salser. Probes for the HLA-DR α chain were pHLA DR α and p-II-α-1, generously provided by B. Mach and colleagues[25] and D. Larhammar and colleagues,[26] respectively. The probe for the HLA-DQ α chain was pDCH1, generously provided by C. Auffray and co-workers.[27] With our hybridization and rinsing conditions, the probes for HLA-DR α and HLA-DQ α bind to distinct populations of mRNA.[28,29]

Immunofluorescence. Expression of HLA-D antigens on individual cells was determined by indirect immunofluorescence after exposure of cells to murine monoclonal antibodies and fluorescein-coupled $(\text{FAb}')_2$ anti-mouse antibody (FITC-anti-mouse). The murine monoclonal antibodies used in this study have been well characterized. The L243 antibody (IgG2a)[30] (generously provided by T. Meeker and R. Levy) reacts with a nonpolymorphic region of the HLA-DR antigen. The Leu 10 antibody (IgG1)[31] (obtained from Becton Dickinson, Mountain View, CA) reacts with the HLA-DQ antigen on cells with DR allotypes 1,2,4,5,6,8,9, and w10.[32] Control antibodies were a murine myeloma IgG2a protein (Coulter Diagnostics, Hialeah, FL) that does not react with human cells and Leu 5 (IgG1, Becton Dickinson) that does not bind to myeloid cells. The FITC anti-mouse antibody was obtained from Cappel Laboratories (Downington, PA).

Cells were prepared for immunofluorescence studies by washing in Dulbecco's phosphate-buffered saline (PBS) containing 2% heat-inactivated fetal calf serum (FCS) and 0.1% azide (PBS-A), at 4°C. Aliquots of 1 to 3 × 10^6 cells were incubated with murine monoclonal antibody, diluted in 100 μL of PBS-A, for one hour at 4°C, rinsed three times in PBS-A, and then incubated with FITC anti-mouse antibody for 30 minutes. Cells were washed in PBS-A, and immunofluorescence was measured. The following measurements were obtained for each sample of cells: autofluorescence, immunofluorescence after exposure to FITC anti-mouse antibody alone, indirect immunofluorescence after exposure to a control murine monoclonal antibody, and indirect immunofluorescence after exposure to a murine monoclonal antibody directed against one of the HLA-D antigens. Specific binding of antibodies directed against the HLA-DR antigens was determined in relation to binding of the control murine monoclonal antibody of the same isotype. Autofluorescence and binding of FITC anti-mouse antibody alone was always equal to or less than the binding of control antibody. Each experiment was repeated two to four times. In pilot experiments, binding of the Leu 5 control antibody to myeloid cells was not different than binding of the FITC anti-mouse antibody alone. Therefore, binding of the Leu 10 antibody was measured relative to the FITC anti-mouse antibody.

Preliminary saturation studies were performed on ME and Raji cells (Table 1), and showed that an antibody dilution of 1:20 of either Leu 10 or L243 (10 μg/mL) was sufficient to produce maximal cell fluorescence. This concentration of primary antibody was used in all experiments. The second antibody was also used at saturating concentrations (100 μg/mL).

Table 1. Indirect Immunofluorescent Measurement of HLA-DR and HLA-DQ

Saturation Studies			
Antibody	Concentration (μg/mL)	Log Peak Immunofluorescence	Cells Binding to Antibody (%)
A: Binding of primary antibody to T lymphocyte line (ME)			
Leu 10	2	114	56
	4	124	60
	10	143	74
	20	148	61
L243	2	158	75
	4	181	69
	10	193	61
	20	189	67
B: Binding of second antibody (FITC antimouse antibody) to B lymphocyte line (Raji)*			
FITC antimouse Ab	10	144	69
	30	145	75
	100	157	76
	300	157	79

*Raji cells were preincubated with L243 antibody, 10 μg/mL.

The effect of blocking the Fc receptors with excess human IgG was studied using HL-60 cells induced to differentiate with DMSO and γ-IFN.[33] Aliquots of 10^6 cells were incubated in a volume of 100 μL with L243 alone [50 μg/mL (300 nm)], or L243 followed by human IgG (Cappel)[2,400 μg/mL (15 μmol/L)]. Each incubation was for one hour at 4°C, and cells were rinsed three times in PBS-A after each incubation. Cells were then incubated with FITC anti-mouse antibody, and immunofluorescence was measured.

Flow cytometry. Immunofluorescence was analyzed on the EPICS V Cell Sorter (Coulter Electronics) coupled with an Innova 90 Laser (Coherent). Samples were excited with the 488 γ wavelength, and fluorescence was obtained on a minimum of 20,000 cells gated from the forward-angle light-scatter profile.[34] Dead cells were excluded from analysis by setting an appropriate threshold trigger on low forward-angle light-scattering.[35] The distribution of cells as a function of the log fluorescence intensity was determined for each sample of cells. Because of differences in autofluorescence, in amounts of antigen expressed, and in percentage of positive cells in the various samples, the cytofluorometric data were also normalized to allow comparison of the relative levels of antigen expression. Normalization involved converting the data to a linear function, determining the median from the distribution, and multiplying this by the percentage of positive cells in each population. In this phase of the study, fluorescence of cells exposed to FITC goat anti-mouse antibody alone was considered background. This method gives a quantitative estimate of the level of antigen expression by a population of cells[36] (Tables 2 and 3).

Pulse-chase labeling of HLA-DR antigens. Raji or KG-1 cells were labeled by incubation for three hours with ^{35}S methionine (100 μCi/mL, 350 mCi/mmol/L, Amersham, Arlington Heights, IL) in methionine-deficient α medium (α-modified Eagle's medium, Flow Laboratories). Cells were washed three times in α medium containing 2 mmol/L of unlabeled methionine and resuspended at a density of 10^6 cells in α medium containing 10% FCS and 1 mmol/L of methionine. Cells were harvested at various times, and the incorporation of label into HLA-DR antigens was measured by immunoprecipitation and autoradiography.

Immunoprecipitation of HLA-DR antigens was performed by

Table 2. Expression of HLA-DR and HLA-DQ Antigens on Human Lymphoid Cells

Cell Name	Human Cell Type	Median Fluorescent Intensity (Log Scale)	Fluorescent-Positive Cells (%)	Antigen Intensity of Population*
A: HLA-DR antigen expression				
Raji	Burkitt's (B lymphocyte)	136	85	6,715
P3HR-1	Burkitt's (B lymphocytes)	131	80	5,376
ME	T lymphocytes infected with HTLV-1	151	83	10,092
ME-II	B lymphocytes infected with HTLV-1	156	86	12,109
—	PB T lymphocytes	0	0	0
—	Mitogen stimulated PB T lymphocytes	93	85	2,040
B: HLA-DQ antigen expression				
Raji		53	65	468
P3HR-1		103	60	1,728
ME		106	82	2,624
ME-II		123	67	3,859
PB T lymphocytes		0	0	0
Mitogen-stimulated PB T lymphocytes		34	53	200

*Data derived by linearizing median log fluorescent intensity and multiplying that number by the percentage of fluorescent-positive cells.

HTLV-1, human T cell leukemia virus; PB, peripheral blood.

making cell lysates, incubating cell lysates with L-243 antibody, and precipitating immune complexes with protein A agarose (Sigma, St Louis.[37] To make the cell lysates, the labeled cells were resuspended in lysing buffer [0.4% NP-40, 0.4% Na deoxycholic acid, 300 mmol/L of NaCl, 10 mmol/L of EDTA, 100 mmol/L of Tris (pH 7.5), 2 mg/mL of bovine serum albumin (BSA), and 1 mmol/L of phenylmethylsulfonylfluoride, PMSF), and mechanically disrupted. Lysates were centrifuged 10 minutes at 12,000 g, 4°C, and diluted with an equal volume of immunoprecipitation buffer (PBS containing BSA 10 mg/mL, methionine 2 mg/mL, *N*-lauroyl sarcosine 0.1%, pH 7.4). Samples were cleared by mixing with 20 μL of a suspension (1:1) of (protein A agarose:immunoprecipitation buffer), incubating for 15 minutes at 4°C, and centrifuging at 12,000 g for 2 minutes. The supernatant was mixed with 10 μg of L-243 antibody and incubated overnight at 4°C with gentle rocking. Immune complexes were immunoprecipitated by adding protein A agarose to the samples, incubating for 30 minutes, and centrifuging for two minutes at 12,000 g. The pellet was washed five times in PBS containing 0.1% *N*-lauroyl sarcosine, pH 7.4. Immune complexes were denatured by boiling for five minutes in SDS sample buffer (1% SDS, 10% β-mercaptoethanol, 62.5 mmol/L of Tris (pH 6.8), 20% glycerol, bromphenol blue), and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).[38] Electrophoresis was performed on a 10% polyacrylamide gel containing SDS, at a constant

Table 3. Expression of HLA-DR and HLA-DQ on Human Myeloid Cells

Cell Name	Human Cell Type	Median Fluorescent Intensity (Log Scale)	Fluorescent-Positive Cells (%)	Antigen Intensity of Population*
A: HLA-DR antigen expression				
KG-1a	Early myeloblast	52	85	525
KG-1	Myeloblast	120	77	3,942
ML-3	Myelomonoblast	23.4	36	115
HEL	Bipotent	97.5	95	2,141
HL-60	Promyelocyte	0	—	0
—	PBM	131	92	6,203
—	PAM	74	79	1,106
—	Granulocytes	0	—	0
—	Granulocytes-γ-IFN	0	—	0
B: HLA-DQ antigen expression				
KG-1a	—	40	12	38
KG-1	—	39	11	54
ML-3	—	0	—	0
HEL	—	0	—	0
HL-60	—	0	—	0
—	PBM	67	23	282
—	PAM	20	23	64
—	Granulocytes	0	—	0
—	Granulocytes-γ-IFN	0	—	0

HEL, cells are bipotent erythroblasts/monoblasts; PBM, peripheral blood monocytes; PAM, pulmonary alveolar macrophages; granulocytes + γ-IFN, peripheral blood granulocytes incubated with 500 to 1,000 U/mL of γ-interferon.

*Data derived by linearizing median log fluorescent intensity and multiplying that number by the percentage of fluorescent positive cells.

current of 20 mA. Gels were prepared for fluorography with ENHANCE (New England Nuclear, Boston) according to the manufacturer's instructions. Autoradiograms were performed as described above, except that ^{35}S autoradiograms were performed with preflashed X-ray film. The intensity of protein bands was quantitated by laser densitometry.

Cells and culture conditions. Myeloid and lymphoid cell lines were cultured in α medium supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA) and 100 U/mL of penicillin-streptomycin (Irvine Scientific), in a 5% CO_2 atmosphere. Cultures were divided every five days to maintain a density $<10^6$ cells/mL. Pulmonary alveolar macrophages (PAM) and peripheral blood cells were obtained from normal volunteers. Peripheral blood mononuclear cells and polymorphonuclear leucocytes were isolated by buoyant-density centrifugation.[39] To enrich for either PAM or PBM, cells were placed in 75-cm^2 plastic flasks containing α medium with 10% FCS. Nonadherent cells were removed from the flasks after four and 24 hours of culture. More than 95% of the adherent cells contained strong α-naphthyl acetate esterase activity, an enzyme that exists in monocytes and macrophages but not in lymphocytes or granulocytes. The T lymphocytes were isolated from the mononuclear fraction of peripheral blood by sheep E rosetting.

Mitogen stimulation of peripheral blood T lymphocytes was performed by incubating cells with 1% phytohemagglutinin (PHA, Burroughs Welcome, Triangle Park, NC) for 48 hours. Interferon treatment of cells was performed by incubating cells with recombinant human γ-IFN (generously provided by Genentech, South San Francisco) at 500 U/mL for 72 hours. Cells from myeloid lines were induced to differentiate terminally in vitro by exposure to 1,25 dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$, 5×10^{-8} mol/L] (generous gift of M. Uskokovic, Hoffmann-La Roche, Nutley, NJ) for five days[40] or by exposure to 1.25% DMSO (Sigma) for five days.[41] Aliquots of the differentiated cells were incubated with γ-IFN for 72 hours. Viability of cells, assessed by trypan blue exclusion, was 65% to 90% after induction of terminal differentiation.

Prior studies showed that the Leu 10 antibody fails to react with HLA-DQ antigens on cells that are homozgous for the DR3 or DR7 HLA-DR allotypes.[31] The DR allotypes of cell lines used in this study were previously reported:[32] HL-60, DR 2,7; U-937, DR 4,6; Raji, DR3,6. The DR allotype of cell lines Me (DR 7,8) and KG-1 (MT2, associated with DR 3,5, W6) were determined by a microcytotoxicity assay.[42]

RESULTS

Immunofluorescent studies of expression of the HLA-DR and HLA-DQ antigens. Expression of HLA-DR and HLA-DQ antigens was determined by exposing cells to the murine monoclonal antibodies L243 and Leu 10, respectively, and measuring specific binding by indirect immunofluorescence. These monoclonal antibodies were previously used to study HLA-D antigen expression on lymphoid and myeloid cells.[7,9,15,16,31,32,43] Expression of HLA-DR and HLA-DQ antigens could be detected on cells from each of the human B and T lymphocyte cell lines (Fig 1, Table 2). More than 60% of each cell population expressed both antigens. The intensity of binding of the Leu 10 antibody was consistently less than that of the L243 antibody. Expression of both MHC class II antigens was most intense on T and B cells infected with the HTLV-I, in comparison to other lymphoid cells.

The myeloid cell populations expressed HLA-DR antigens to a variable extent (Fig 2, Table 3). Cell lines of immature phenotype including KG-1, KG-1a, and HEL[44,45,46] expressed the HLA-DR antigen on $>$75% of the cells. HLA-DR expression, however, was weak or absent on cells from relatively mature cell lines, such as HL-60 (promyelocytes)[47] or U-937 (monoblasts).[48] Exposure of HL-60 cells to 1,25(OH)$_2$D$_3$ induced morphological and cytochemical evidence of maturation to a macrophage-like phenotype in 90% of the cells, yet induced HLA-DR expression at low levels on only 20% to 40% of the cells (Fig 3).

Expression of the HLA-DQ antigens could not be detected on cells of the majority of the myeloid cell lines (Fig 4, Table 3). Only KG-1 and KG-1a cell lines, which manifest the phenotype of immature and very immature myeloblasts, respectively, expressed HLA-DQ antigens at low intensity on 12% of the cells. Peripheral blood monocytes and pulmonary

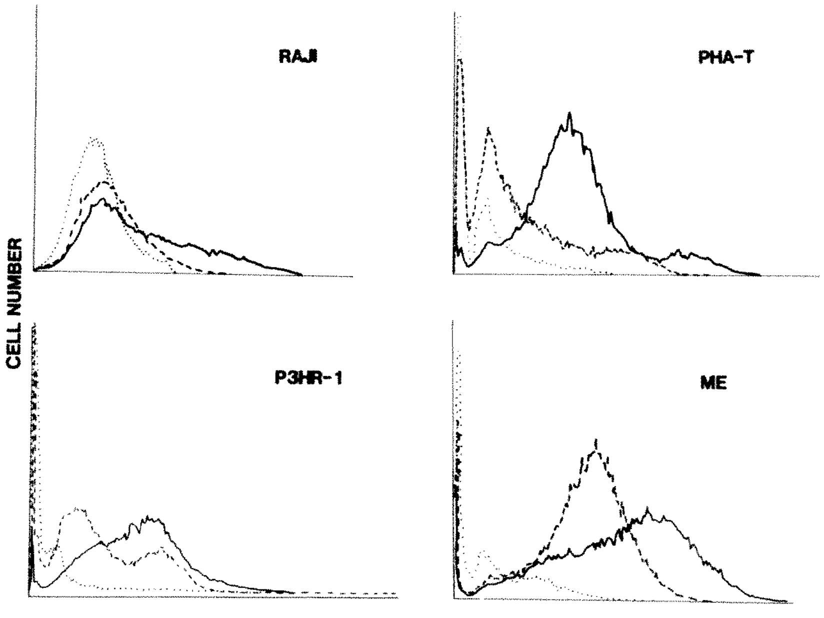

Fig 1. Cytofluorometric analysis of expression of the HLA-DR and HLA-DQ antigens on lymphoid cells. Cells from lymphoid lines (Raji, P3HR-1, ME) or normal human peripheral blood T lymphocytes stimulated with phytohemagglutinin (PHA-T) were incubated serially with murine monoclonal antibodies and fluorescein-conjugated goat anti-mouse antibody (FITC anti-mouse), and fluorescence intensity was measured by flow cytometry. Raji and P3HR-1, B lymphocyte lines derived from Burkitt's lymphoma cells; ME, T lymphocyte line transformed with HTLV-1. Anti-HLA-DR (L243) antibody (—); anti-HLA-DQ (leu 10) antibody (---); control (FITC anti-mouse antibody) (· · ·). Cell number is plotted on a linear scale, and fluorescence intensity is plotted on a logarithmic scale. Cell number on vertical axis is plotted as a function of log fluorescence intensity on the horizontal axis.

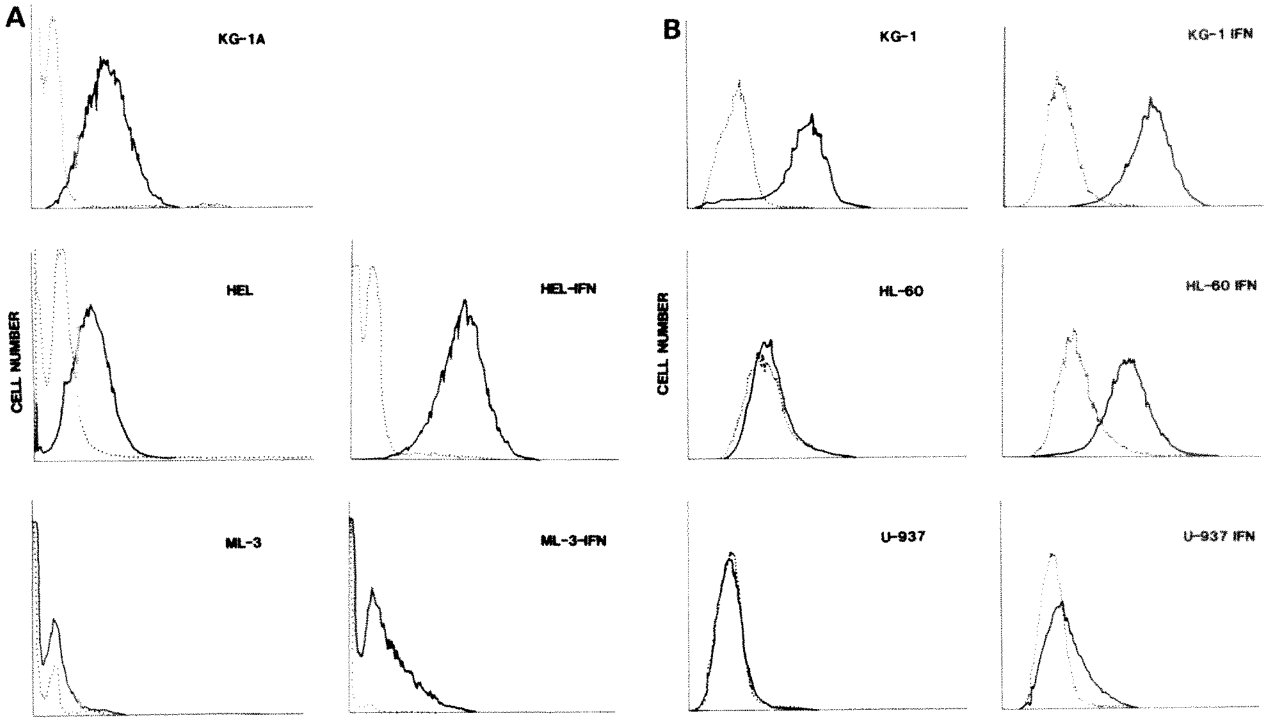

Fig 2. (A and B) Cytofluorometric analysis of expression of the HLA-DR antigen on myeloid cells. Cells from myeloid cell lines were incubated serially with murine monoclonal antibody directed against the HLA-DR antigen (L243) and fluorescein-conjugated goat anti-mouse antibody (FITC anti-mouse), and fluorescence intensity was measured by flow cytometry. Cell number is plotted on a linear scale, and fluorescence intensity is plotted on a logarithmic scale. Cell number on vertical axis is plotted as a function of log fluorescence intensity on the horizontal axis. Cell lines KG-1, KG-1a, HL-60, U-937, HEL, and ML-3 are described in the text. Anti-HLA-DR (L243) antibody (—); control (murine monoclonal antibody (IgG2a) (· · · ·). IFN, cells exposed to recombinant γ-interferon (γ-IFN) 500 U/mL for 72 hours.

alveolar macrophages expressed HLA-DQ antigens on the surface of 25% to 30% of the cells. The HLA-DQ antigens, however, could not be detected on either HL-60 or U-937 cells induced to differentiate to macrophages in vitro by exposure to $1,25(OH)_2D_3$. Likewise, the HLA-DQ antigens could not be detected on peripheral blood granulocytes or on HL-60 cells induced to differentiate to metamyelocytes by exposure to 1.25% DMSO (Table 3).

Modulation of expression of HLA-DR and HLA-DQ by γ-IFN. Exposure to γ-IFN has been reported to increase expression of HLA-DR antigens on myeloid cells, including human fetal and adult peripheral blood monocytes[15-17,43] and cells from the myeloid lines HL-60, ML-1, and U-937.[15,48,49,50] We examined the effect of γ-IFN on expression of HLA-DR and HLA-DQ antigens by cells from a panel of myeloid cell lines that differed in their apparent degree of maturation. Exposure to γ-IFN increased expression of HLA-DR antigens on the surface of KG-1, ML-3, and HEL cells, and induced measurable expression on HL-60 cells (Fig 2). In some experiments, γ-IFN induced expression of HLA-DR on the surface of the U-937 cells. γ-IFN also caused a dramatic increase in expression of HLA-DR antigens on HL-60 and U-937 cells induced to differentiate to macrophagelike cells by exposure to $1,25(OH)_2D_3$ (Fig 3). Paradoxically, HL-60 cells exposed to DMSO expressed HLA-DR when subsequently incubated with γ-IFN. The HL-60 cells exposed to DMSO and γ-IFN morphologically resembled metamyelocytes and did not express nonspecific esterase, the cytochemical marker of macrophages. Peripheral blood granulocytes harvested from normal volunteers did not express HLA-DR antigens after a 48-hour exposure to 500 or 1,000 U/mL of γ-IFN in vitro (Table 3).

Similar to findings previously reported for HL-60 cells,[15] we found that γ-IFN exposure had disparate effects on HLA-DR and HLA-DQ antigen expression on cells of various myeloid lines. Exposure to γ-IFN increased HLA-DQ expression on KG-1 cells (Fig 5). HLA-DQ antigen expression could not be detected on other myeloid cell lines, however, either constitutively or after exposure to γ-IFN (Fig 5). Although HL-60 and U-937 cells exposed to $1,25(OH)_2D_3$ and γ-IFN differentiated in vitro to mature macrophagelike cells, the HLA-DQ antigen could not be detected on these cells.

Exposure of myeloid cells to either γ-IFN or other agents that induce differentiation in vitro increases the expression of Fc receptors.[51,52] Although murine IgG2a binds to the human Fc receptor,[53,54] the binding of L243 (IgG2a) to myeloid cells could not be attributed to Fc binding for several reasons. First, binding of L243 to the cells was significantly higher than binding of control IgG2a antibody to the same cell population in all cases. Binding of the control antibody was congruent with the background fluorescence (FITC antibody

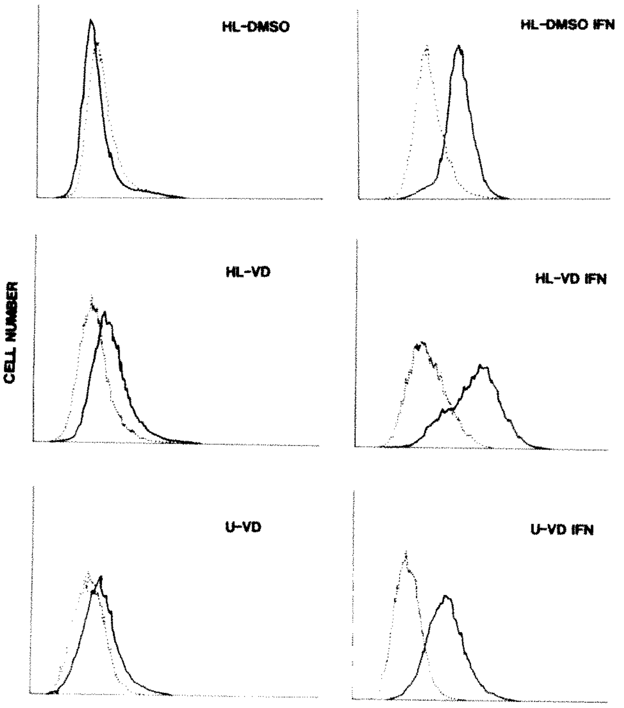

Fig 3. Cytofluorometric analysis of expression of the HLA-DR antigen on myeloid cells induced to differentiate in vitro. Cells from myeloid cell lines HL-60 and U-937 were induced to differentiate in vitro by exposure to either dimethyl sulfoxide (DMSO) or 1,25 dihydroxyvitamin D_3 (VD). Expression of HLA-DR antigen was measured as in Fig 2. Anti-HLA-DR (L243) antibody (—); control (murine monoclonal antibody, IgG2a) (· · ·). HL-DMSO and HL-VD, HL-60 cells induced to differentiate with DMSO or 1,25(OH)D_3, respectively; U-VD, U-937 cells induced to differentiate with 1,25(OH)D_3; IFN, cells exposed to recombinant γ-IFN 500 U/mL for 72 hours. Cell number on vertical axis is plotted as a function of log fluorescence intensity on the horizontal axis.

alone) in all cases except with the differentiated HL-60 cells, and, in these cells, the binding of L243 was substantially above that of the control antibody. Second, several cell types known to express Fc receptors failed to bind L243: granulocytes, U-937 cells, and DMSO-treated HL-60 cells.[55] Finally, the L243 antibody bound to the surface of differentiated HL-60 cells could not be displaced by subsequent incubation of cells with a 50-fold excess of human IgG (Fig 6).

HLA-DR pulse-chase studies. Almost all samples of lymphoid cells that we examined expressed more HLA-DR than did the myeloid samples. The difference was not secondary to cell surface area because both cell types were similar in size as determined by light microscopy (15 to 20 μm in diameter). The decreased expression of DR could be secondary to either decreased production or decreased survival of these proteins in the myeloid cells as compared with the lymphoid cells. Pulse-chase experiments were performed to determine the relative stability of HLA-DR proteins in and on Raji (lymphoid) cells and KG-1 (myeloid). Cells were pulsed with ^{35}S methionine for three hours, washed, and returned to culture in α medium with added unlabeled methionine for varying lengths of time. As shown in Fig 7 and Table 4, the relative stability of HLA-DR antigens was equivalent on Raji and KG-1 cells, with a half-life (t½) of <24 hours. The intensity of bands corresponding to the α (33 kd) and β (29 kd) chains of the DR antigen was identical in all samples, suggesting coordinated synthesis and expression of these subunits.[55]

Analysis of HLA-DR and HLA-DQ mRNA in myeloid and lymphoid cells. Previous studies using myeloid cells demonstrated that mRNA for HLA-DRα and β chains is expressed in myeloid cells that express HLA-DR antigen, but not in those cells that failed to express HLA-DR.[15] Induction of expression of HLA-DR antigens after exposure to γ-IFN was coincident with an accumulation of HLA-DR mRNA in HL-60 cells and in nonmyeloid cells.[15,56] We investigated the expression of mRNA for the HLA-DRα chain in myeloid and lymphoid cell lines by hybridization of Northern blots with a cDNA probe for the HLA-DR α chain (pDR α1) (Fig 8). The cells from each lymphoid line contained abundant HLA-DR α chain mRNA. This transcript was $\sim$1.4 kilobases (kb), as previously reported.[57,58] The cells of myeloid lines contained mRNA for HLA-DR α chain to a variable extent; no expression was detectable in HL-60 cells that also failed to express the HLA-DR antigen; mRNA levels were highest in KG-1 cells. The mRNA accumulation for DR crudely paralleled the level of antigen expression, with DR mRNA levels highest in HTLV-I-infected T and B lymphocytes (ME and ME-II).[59]

Accumulation of mRNA for HLA-DQ was studied by hybridization of Northern blots to a probe for the HLA-DQ α chain.[60,61] Under our hybridizing and washing conditions, the pDCH1 probe does not cross-hybridize with HLA-DR mRNA. Lymphoid cells expressed two mRNA bands, of 1.1 and 1.4 kb, which hybridized with the HLA-DQ α probe. mRNA for the HLA-DQ α chain could not be detected in the myeloid cell lines KG-1, ML-3, HEL, or HL-60, however. The lack of detectable HLA-DQ mRNA sequences in these myeloid cells probably does not represent a nonspecific degradation of mRNA because (a) the same mRNA pools were used for the HLA-DR and DQ mRNA experiments; (b) the RNA gels showed undegraded 18S and 28S bands; and (c) each filter was stripped of probe and rehybridized with a tubulin cDNA, and all samples had comparable amounts of mRNA for tubulin (data not shown).

DISCUSSION

We previously noted that HL-60 promyelocytic leukemia cells fail to express constitutively the MHC class II antigens but are stimulated to express HLA-DR antigens and mRNA on stimulation with γ-IFN.[15] In the present study, we showed that expression of MHC class II antigens and mRNA in human myeloid leukemia cell lines varies with stage of differentiation. The capacity of cells to express MHC class II antigens after exposure to γ-IFN is also related to the degree of maturation of the cells. Our data provide additional evidence for discoordinate regulation of expression of the HLA-DR and HLA-DQ antigens in human myeloid cells.

The HLA-DR antigens were expressed on all lymphoid lines that we studied, including T and B lymphocyte lines infected with HTLV-I as well as mitogen-stimulated periph-

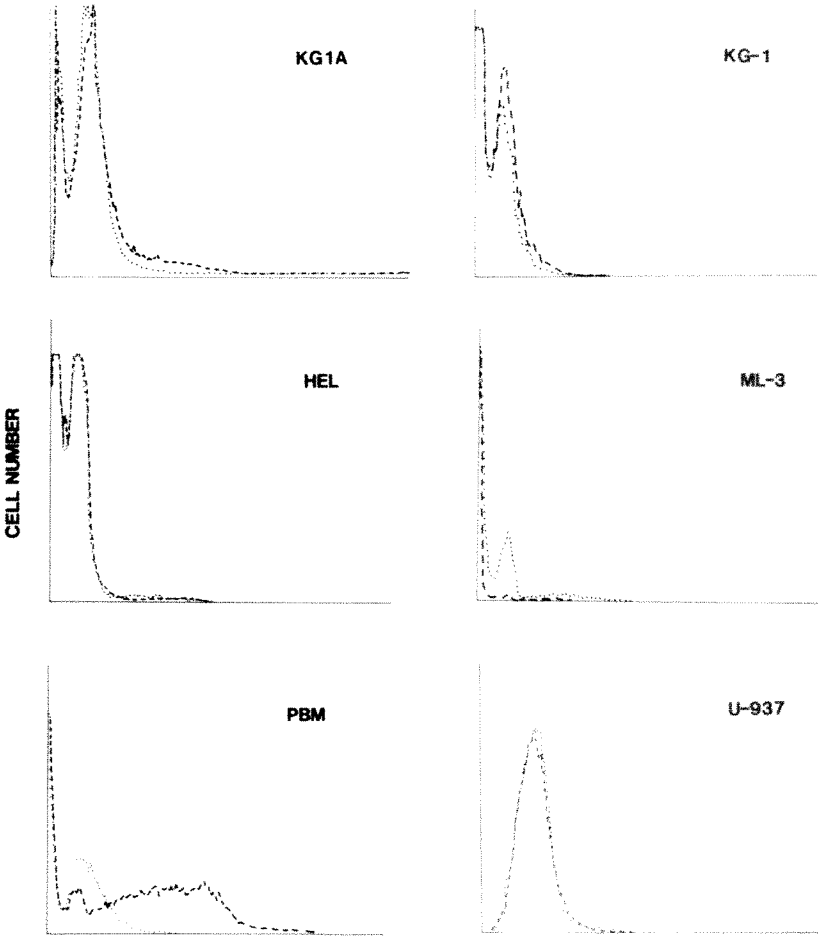

Fig 4. Cytofluorometric analysis of expression of the HLA-DQ antigens on myeloid cells. Myeloid cells from permanent lines or peripheral blood monocytes (PBMs) from normal volunteers were incubated serially with murine monoclonal antibody directed against the HLA-DQ antigen (Leu 10), and fluorescein-conjugated goat anti-mouse antibody (FITC anti-mouse); intensity of fluorescence was measured by flow cytometry. Cell number is plotted on a linear scale, and fluorescence intensity is plotted on a logarithmic scale. Cell number on vertical axis is plotted as a function of log fluorescence intensity on the horizontal axis. Cell lines KG-1, KG-1a, U-937, HEL, and ML-3 are described in the text. Anti-HLA-DQ (Leu 10) antibody (---); control (FITC goat anti-mouse antibody) (· · ·).

eral blood T lymphocytes. In contrast, HLA-DR antigens were expressed to a variable extent on cells from myeloid lines, with high levels of expression on myeloid cell lines with the phenotypes of either myeloblasts (KG-1 and KG-1a) or bipotent-erythroblasts/myeloblasts (HEL). Cell lines that manifested a greater degree of myeloid maturation, such as ML-3, HL-60, and U-937, expressed HLA-DR antigen at low or negligible levels. Although peripheral blood monocytes and pulmonary alveolar macrophages expressed HLA-DR antigens at high levels on 85% to 100% of the cells, terminal differentiation of HL-60 and U-937 to macrophagelike cells in vitro induced expression of HLA-DR antigen at a low intensity on only 25% to 50% of cells.

The HLA-DQ antigens were detectable on all lymphoid cells with the exception of peripheral blood T lymphocytes. The intensity of binding of the Leu 10 antibody was less than that of L243 on each cell line. This may indicate a lower level of expression of HLA-DQ antigens on these cells. Although the antibodies are of different isotype and may differ in affinity for their respective antigens, the antibodies were used in saturating concentrations. Under these conditions, the level of antibody binding is proportional to antigen expression.[8,62] Similarly, the FITC anti-mouse antibody was present at saturating levels. Other investigators have reported that antigen density of HLA-DQ was apparently less than that of HLA-DR on human monocytes.[8]

The KG-1 and KG-1a myeloblast cells weakly expressed HLA-DQ antigens on a low percentage of cells. The HLA-DQ antigens could not be detected on other myeloid cell lines of more differentiated phenotype. HLA-DQ expression was measurable on 25% to 30% of peripheral blood monocytes and pulmonary alveolar macrophages, however. Our inability to detect DQ expression on myeloid cell lines cannot be explained by the allotype specificity of the Leu 10 antibody because several of the myeloid cell lines have been typed for HLA-DR and are not homozygous for DR3 or DR7. Conversely, the binding of Leu 10 to lymphoid cells probably cannot be attributed to artifactual binding to cross-reactive non-DQ antigens. In serial immunoprecipitation studies of lymphoblastoid cells, Leu 10 specifically immunoprecipitated HLA-DQ polypeptide chains.[31] Our results provide further evidence that HLA-DR antigen expression may be regulated independently of that of HLA-DQ in myeloid cells. Several investigative groups have reported that monocytes from peripheral blood express HLA-DQ antigens at lower levels than HLA-DR, and that 50% to 75% of monocytes are negative for HLA-DQ by immunofluorescence.[7,8,16,17] Discoordinate expression of DR and DQ antigens has also been described in studies of hematopoietic progenitor cells and leukemic blast cells. Granulocyte-macrophage progenitor cells (GM-CFC) from normal human bone marrow expressed HLA-DR in studies using FACS and complement-

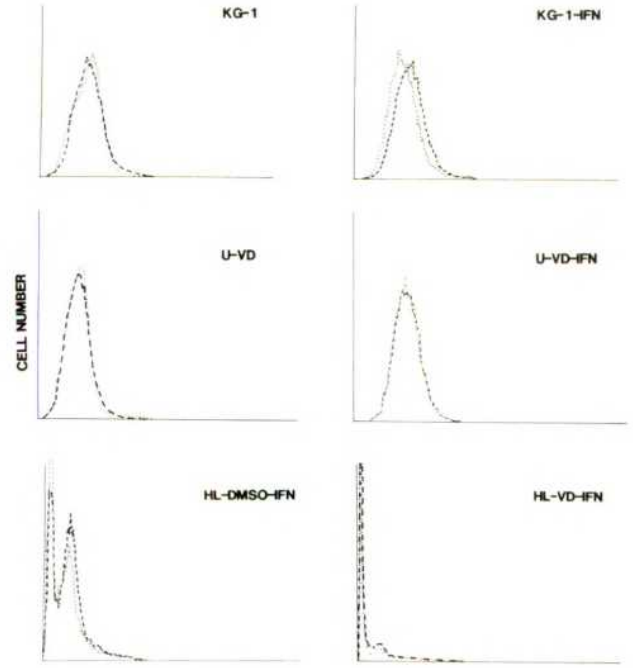

Fig 5. Cytofluorometric analysis of expression of the HLA-DQ antigens on myeloid cells: effect of γ-interferon (γ-IFN). Myeloid cells from lines were cultured in α medium for 72 hours with and without γ-IFN. Expression of HLA-DQ antigen was determined as described in the legend for Fig 5. Anti-HLA-DQ (leu 10) antibody (---); control (FITC goat anti-mouse antibody) (· · ·). IFN, cells exposed to recombinant γ-IFN 500 U/mL for 72 hours. Cell number on vertical axis is plotted as a function of log fluorescence intensity on the horizontal axis.

mediated cytotoxicity.[9] When marrow cells were incubated with antibody directed against HLA-DQ and sorted by FACS, however, GM-CFC were found only in the HLA-DQ–negative fraction.[9] Two investigative groups noted that expression of HLA-DR antigen could be measured on most leukemic blast cells, whereas HLA-DQ antigen was limited to a subset of the leukemic cells.[63,64]

The level of expression of the HLA-DR and HLA-DQ antigens is probably in part regulated at a pretranslational level. Low or undetectable levels of mRNA for the HLA-DR α and HLA-DQ α chain were noted in cells that expressed negligible levels of the corresponding antigens. The level of HLA-DRα mRNA expression was markedly lower in myeloid cells as compared with lymphoid cells, yet levels of HLA-DR antigen expression varied to a much smaller extent. This discrepancy could not be attributed to degradation of the myeloid RNA, nor did it represent decreased stability of the HLA-DR antigen on myeloid cells. The turnover of HLA-DR antigen after metabolic labeling was similar in myeloid and lymphoid cells. Prior studies using melanoma cell lines noted a discrepancy between the level of expression of mRNA for the HLA-DR antigens and surface membrane expression of the HLA-DR antigens.[56] Messenger RNA "run-on" and stability experiments will help to elucidate this discrepancy.

The level of binding of the cDNA probes for HLA-DR to RNA blots should provide an accurate indication of the level

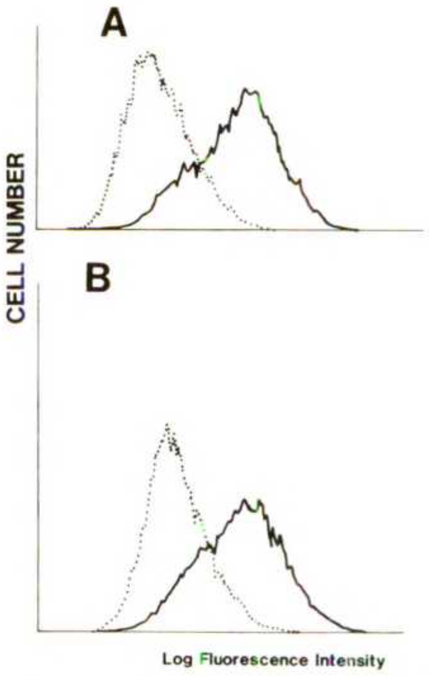

Fig 6. Cytofluorometric analysis of expression of the HLA-DR antigen on myeloid cells: effect of Fc receptor blockade with human IgG. HL-60 cells were induced to express HLA-DR antigen by incubation with dimethylsulfoxide (DMSO) and γ-IFN. Cells were incubated with (A) anti-HLA-DR antibody (L243, 300 nmol/L), or (B) with L-243 (300 nmol/L) followed by human IgG [15μmol/L], and HLA-DR expression was measured by flow cytometry after incubation of cells with FITC anti-mouse antibody. Cell number is plotted on a linear scale, and fluorescence intensity is plotted on a logarithmic scale. Anti-HLA-DR (L243) antibody (—); control [murine monoclonal antibody (IgG2a)] (· · ·).

of expression of mRNA for the HLA-DR antigens. The cDNA probes were derived from nonpolymorphic regions of the RNA transcript encoding the DR α chain, and react with α chain RNA from cells of all DR types.[25,26,57] Measurement of DR α chain RNA is also probably an accurate reflection of

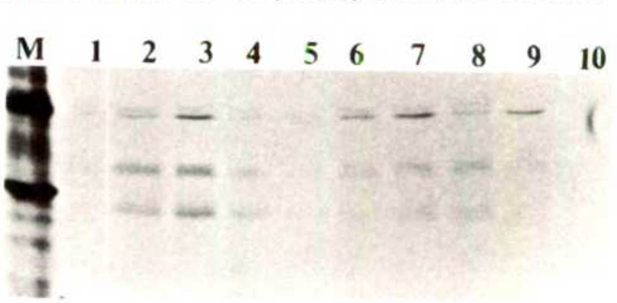

Fig 7. Turnover of proteins for HLA-DR antigen on lymphoid and myeloid cells. Lymphoid (Raji) cells (lanes 1 through 5) and myeloid (KG-1) cells (lanes 6 through 10) were incubated with ^{35}S methionine for three hours, washed, and returned to culture with unlabeled methionine (cold chase). Aliquots were removed after being labeled with ^{35}S-methionine (lanes 1 and 6), and after 3 hours (lanes 2 and 7), 6 hours (lanes 3 and 8), 24 hours (lanes 4 and 9), and 48 hours (lanes 5 and 10) of the chase period. Cells were lysed, and HLA-DR antigen proteins were immunoprecipitated and analyzed as described in the text. Lane M, mol wt standards, 30 kd and 46 kd.

Table 4. Turnover of HLA-DR Antigen Proteins on Lymphoid and Myeloid Cells

	Density of Hybridization Grains on Film	
Duration of Chase Period (h)	Raji Cells	KG-1 Cells
0	140	150
3	1,620	1,170
6	2,350	800
24	780	280
48	70	50

Lymphoid (Raji) and myeloid (KG-1) cells were labeled with ^{35}S methionine and returned to culture with unlabeled methionine (cold-chase) as described in the legend for Fig 7. HLA-DR proteins in cell lysates were analyzed by immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography at −70°C, using preflashed Kodak XAR film and an intensifying screen. The density of silver grains corresponding to protein bands of 29 kd and 33 kd was quantitated with a laser densitometer (Joyce-Leoble) and expressed in arbitrary units.

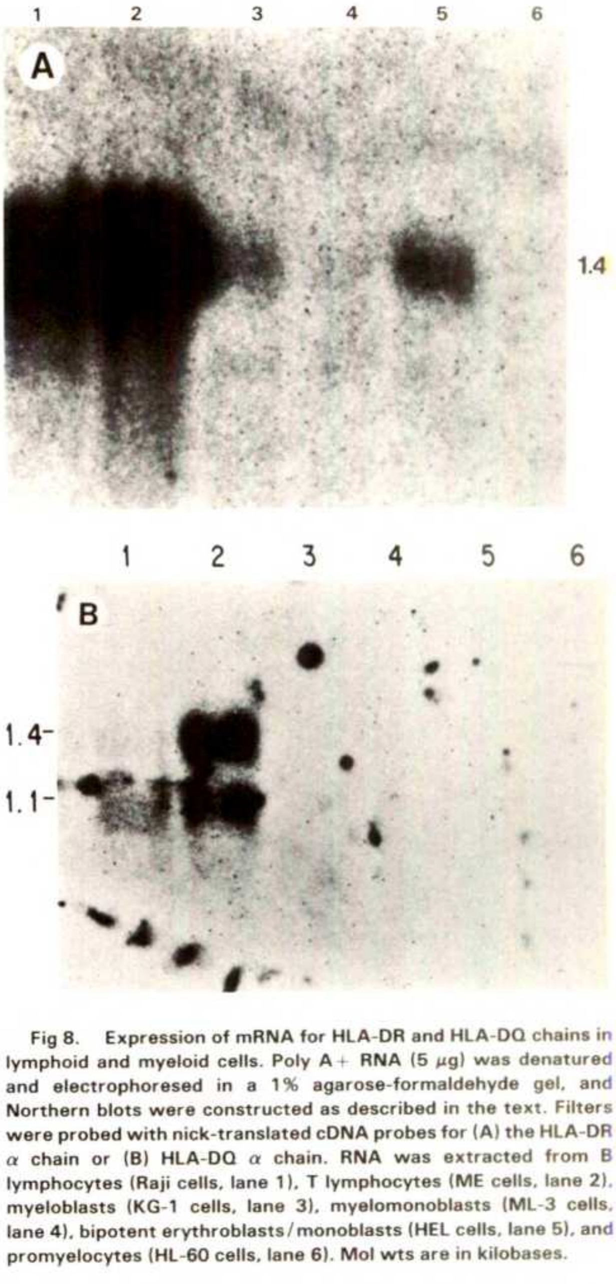

Fig 8. Expression of mRNA for HLA-DR and HLA-DQ chains in lymphoid and myeloid cells. Poly A+ RNA (5 μg) was denatured and electrophoresed in a 1% agarose-formaldehyde gel, and Northern blots were constructed as described in the text. Filters were probed with nick-translated cDNA probes for (A) the HLA-DR α chain or (B) HLA-DQ α chain. RNA was extracted from B lymphocytes (Raji cells, lane 1), T lymphocytes (ME cells, lane 2), myeloblasts (KG-1 cells, lane 3), myelomonoblasts (ML-3 cells, lane 4), bipotent erythroblasts/monoblasts (HEL cells, lane 5), and promyelocytes (HL-60 cells, lane 6). Mol wts are in kilobases.

expression of mRNA for the DR β chain, because previous studies demonstrated coordinate expression of α and β chain mRNA and polypeptides in human cells.[15,55,58,65] The pattern of binding to the DQ α probe, pDCH1, is less predictable. At least two genes homologous to DQ α have been identified in the human genome.[61,66] In addition, the expression of the known alleles of DQ are in linkage disequilibrium with specific DR allotypes. We used a cDNA probe homologous to the gene for the α chain of the DC 4 allele, a DC gene that is in linkage disequilibrium with DR allotypes DR4, DR7, and DR9. This probe reacts with RNA from Raji cells (DR3, DR6).[66] In our study, the DC α probe reacted with RNA from Raji cells and ME cells (DR7, DR8). Because the HL-60 cells express DR2 and DR7 and therefore should express DQ4,[32,60,61] the failure to detect DQ α RNA from HL-60 cells probably cannot be attributed to the allotype specificity of the DQ α probe. Two transcripts that hybridized with the PDCH1 probe were noted in Raji cells and ME cells. This pattern of expression was previously noted in RNA from Raji cells[66] and in human endothelial cells[58] and was due to alternative RNA splicing patterns in the 3′ untranslated region of the mature DC α transcript.

The relatively higher level of HLA-DR antigen expression in lymphoid cells as compared with myeloid cells could not be accounted for by changes in the stability of the HLA-DR antigen in these cells. We noted a t½ of <24 hours, similar to the t½ of the I-A antigen on murine macrophages cultured in vitro.[67,68] In contrast, human endothelial cells and dermal fibroblasts continue to express HLA-DR antigens at high levels for >96 hours after withdrawal of γ-IFN.[58] The differences in turnover rate between hematopoietic cells and dermal fibroblasts may reflect the growth rate of the cells, the culture conditions, or tissue-specific variation in antigen stability.

We studied the effect of γ-IFN on HLA-DR and HLA-DQ expression because this lymphokine is known to activate myeloid cells in the immune response. Secretion of γ-IFN by T lymphocytes stimulates parameters of myeloid function, such as interleukin-1 (IL1) synthesis, which are essential for antigen presentation.[13,14] γ-IFN also restores expression of MHC class II antigens on human monocytes cultured in vitro, induces expression of HLA-DR antigens on HL-60 cells, and induces differentiation of myeloid cell lines toward a monocytic phenotype.[7,15-17,69,70] We demonstrated that γ-IFN increases expression of HLA-DR antigens on the surface of several myeloid cell lines. This response to γ-IFN was heightened when HL-60 cells were induced to differentiate terminally with 1,25(OH)$_2$D$_3$. Similarly, U-937 cells, which expressed low to unmeasurable levels of HLA-DR antigen after exposure to γ-IFN alone, were induced to express DR antigens by sequential treatment with 1,25(OH)$_2$D$_3$ and γ-IFN. Other investigators showed that HLA-DR expression could be induced by γ-IFN in U-937 cells pretreated with 5-azacytidine.[49]

HL-60 cells induced to differentiate with DMSO expressed DR antigens after subsequent exposure to γ-IFN. The intensity of expression and the percentage of cells that expressed DR was markedly higher than that seen after exposure of uninduced HL-60 promyelocytes to γ-IFN. Exposure of HL-60 to DMSO alone induced maturation to metamyelocytes as determined by morphology but failed to induce expression of HLA-DR antigens. Because DMSO induces HL-60 cells to express many of the phenotypic and cytochemical characteristics of granulocytes, we examined the expression of HLA-DR on normal human granulocytes exposed to γ-IFN in vitro and found that these cells had undetectable HLA-DR expression. This result suggests that cells from cell lines induced to differentiate in vitro do not bear a strict resemblance to their terminally differentiated counterparts in vivo.[71] In previous reports, DMSO-treated HL-60 cells functioned as accessory cells in T cell stimulation despite their absence of HLA-DR antigen expression.[14] In contrast, HL-60 promyelocytes were not able to function as accessory cells.

The HLA-DQ expression could not be detected on myeloid cell lines after γ-IFN exposure. The only exception to this pattern occurred in KG-1 cells, in which γ-IFN led to a slight, but consistent, increase in the expression of HLA-DQ antigen. In our earlier study, we noted that HL-60 cells did not express HLA-DQ antigens or mRNA after exposure to γ-IFN.[15] Recently, Nunez and colleagues reported that treatment with T cell-conditioned medium failed to induce expression of HLA-DQ in HLA-DQ(−) monocytes.[17] Gonwa and Stobo noted that γ-IFN restored the expression of DR α and DQ α on monocytes maintained in culture. They did not determine whether γ-IFN induced DQ expression de novo or served to restore expression of DQ α on cells that had previously been DQ positive.[7] Basham and Merigan also reported that γ-IFN induced expression of DR and DQ by monocytes, but they noted increased expression of DQ on monocytes maintained in culture, independent of IFN.[16] Collins and co-workers[58] reported that γ-IFN induced expression of HLA-DQ, DR, SB antigens, and mRNAs in human vascular endothelial cells and in dermal fibroblasts in vitro.

Three conclusions are suggested by these studies: (a) The expression of HLA-DQ and HLA-DR may be discoordinate in myeloid cell lines and in freshly isolated human cells of myeloid lineage; (b) exposure of monocytes to γ-IFN increases expression of both HLA-DR and HLA-DQ on the cells, but does not cause most HLA-DQ(−) cells to manifest the DQ antigen; and (c) expression of DR and DQ antigens is labile and may vary with culture conditions. Our inability to detect DQ antigens and mRNA from cells of most myeloid lines probably cannot be ascribed to the reagents used to measure expression of antigen and mRNA, for the reasons noted above. Failure to induce DQ expression may be a function of culture conditions. Both Gonwa and Stobo[7] and Basham and Merigan[16] cultured cells in autologous serum; we used fetal calf serum. The type of serum used to culture murine and human myeloid cells can significantly influence functional properties and expression of MHC class II antigens.[12,72,73] In our study, myeloid cell lines induced to differentiate to macrophagelike cells in vitro failed to express DQ antigens before or after exposure to γ-IFN, in contrast to normal monocytes and macrophages. This finding suggests that factors in addition to γ-IFN and differentiation state of the cell may regulate DQ expression.

The pattern of expression of human MHC class II antigens by myeloid cells appears to be different from the expression of MHC class II antigens by murine myeloid cells. Most murine macrophages express I-A–encoded antigens, which are structural homologues to the human HLA-DQ antigens. Only a minor population of murine macrophages display I-E–encoded antigens (DR homologues).[67] Murine γ-IFN induces expression of I-A mRNA and antigen on murine macrophages,[74,75] induces expression of I-A mRNA in cells of the murine macrophage line P388D,[76] and induces expression of I-A and I-E antigens on another murine myeloid cell line, WEHI-3.[77]

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Mutations in N-*ras* Predominate in Acute Myeloid Leukemia

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Using synthetic oligomers we investigated fresh samples of acute myeloid leukemia (AML) for the presence of mutated *ras* oncogenes. Our original results showed that five of eight samples contained a mutation in codon 13 of the N-*ras* gene. In a subsequent study involving 37 samples, we found only one N-*ras*-13 mutation, and, in addition, mutations in codon 61 of the N-*ras* gene in four cases and a mutation in codon 12 of the Ki-*ras* gene in two cases. Amplification of *ras* genes was not observed. We conclude that in ~20% to 25% of AML cases, a mutated *ras* oncogene is present, predominantly the N-*ras* gene. The occurrence of mutations does not correlate with the cytological features of the leukemia.

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THE HUMAN *ras* family consists of three members, the Ha-*ras*, Ki-*ras*, and N-*ras* genes. They encode homologous proteins of 21 kd, whose cellular location is at the inner surface of the plasma membrane.[1,2] The 21-kd Ha-*ras* protein binds guanine nucleotides and exhibits GTPase activity.[3,4] In this respect, the protein resembles the "G proteins," which are known to be involved in the transduction of receptor-mediated external signals into the cell. A recent study showed that cells injected with *ras*-specific monoclonal antibodies, which presumably inactivate the Ha-*ras* protein, were incapable of entering S-phase of the cell cycle. This suggests that the Ha-*ras* protein plays an essential role in the cell cycle.[5]

In 10% to 20% of the human tumors or tumor-derived cell lines tested, one of the *ras* genes is activated. These activated genes differ from their normal counterparts by their ability to convert the mouse NIH 3T3 cell line into tumorigenic cells.[6] This difference is caused by single point mutations in the coding sequence of the *ras* genes. These mutations occur either in codon 12 or 61 of one of the *ras* genes[7] or in codon 13 of the N-*ras* gene.[8] At least in one case, these mutations affected the GTPase activity of the protein product.[3,4] The current hypothesis is that normal *ras* proteins bind GTP in response to some receptor-mediated signal and, in doing so, transduce the signal further into the cell. Signal transmission would terminate with the intrinsic GTPase activity converting GTP to GDP. In mutated *ras* proteins, the GTPase activity is reduced, resulting in prolonged binding of GTP and hence prolonged activation. In this way, the proper functioning of the signaling pathway in which *ras* proteins are involved is disturbed. Because the *ras* proteins most likely play an important role in cell cycle control, changes in this activity conceivably contribute significantly toward uncontrolled growth, as occurs in tumor cells. Results of Land and colleagues[9] and Ruley,[10] however, showed that additional changes are required in the process of carcinogenesis. These authors established with transfection experiments that primary cell cultures cannot be transformed by an activated Ha-*ras* gene alone, but that an additional "activated" oncogene is required. One of these oncogenes is the c-*myc* gene, which also plays a role in the control of the cell cycle.[11]

To assess the significance of *ras* gene mutations in human tumorigenesis, we are investigating the occurrence of these mutations in fresh samples from human malignancies. For this purpose, we set up a rapid screening assay using synthetic oligonucleotide probes.[8,12,13] In this report, we describe the analysis of 37 samples of acute myeloid leukemia (AML). As reported earlier, five of eight samples analyzed previously contained an N-*ras* mutation in codon 13.[8] In the present 37 samples, we found 7 *ras* gene mutations, of which only one is in codon 13 of the N-*ras* gene.

MATERIALS AND METHODS

Materials. Samples of leukemic cells were taken from peripheral blood or bone marrow and purified over Ficoll gradients. The cells were stored either in liquid nitrogen or in 70% ethanol at 4 °C.

DNA isolation. DNA was isolated as described by Davis and colleagues.[29] Cells were lysed in 500 μL of 0.5% sodium dodecyl sulfate (SDS), 0.1 mol/L of NaCl, 50 mmol/L of Tris-Cl pH 8.0, and 1 mmol/L of EDTA, and incubated 30 minutes at 65 °C with 100 μg/mL of proteinase K; 75 μL of 8 mol/L of potassium acetate was added, and the incubation was allowed to proceed for 15 minutes at 0 °C. The precipitate generated was spun down in an Eppendorf centrifuge, and the supernatant was extracted with 750 μL of chloroform. Subsequently, the DNA was precipitated with ethanol and dissolved in 150 to 300 μL of 10 mmol/L of Tris-Cl pH 8.0 and 0.1 mmol/L of EDTA.

Synthetic oligonucleotide probes. The oligomers were synthesized by G. Veeneman and J. H. van Boom (Organic Chemistry Department, University of Leiden) by the solid-phase triester method. ^{32}P Labeling of the oligomers was obtained by a primer extension reaction using an 8-mer primer as described.[12]

Oligomer hybridizations. DNA was digested with suitable restriction enzymes under the conditions suggested by the supplier (*Pst*I to detect mutations in the N-*ras* gene and the Ki-*ras* gene, *Sst*I for mutations in the Ha-*ras* gene) and fractionated by electrophoresis in 0.5% agarose. The gel was treated as described[12] to denature the DNA in situ and to immobilize the DNA by drying the gel. The dried gels were incubated with the different oligomer probes in 5 × SSPE (1 × SSPE = 10 mmol/L of sodium phosphate pH 7.0,

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0.18 NaCl, and 1 mmol/L of EDTA), 1% SDS, and 100 μg/mL of denaturated salmon sperm DNA for 16 hours at the following temperatures: oligomer probes from the N12 and K12 group (Table 1) at 53 °C, oligomers of the N61 and K61 group at 50 °C, oligomers of the H12 group at 60 °C, and oligomers of the H61 group at 55 °C. The gels were washed at 2 × SSPE and 1% SDS at room temperature and incubated at 5 × SSPE, 1% SDS for 10 minutes at the following temperatures: gels hybridized with N12 and K12 oligomer probes at 63 °C, with N61 and K61 probes at 59 °C, with H12 probes at 73 °C, and with H61 probes at 67 °C. All individuals have given consent to donate bone marrow and blood.

RESULTS

To analyze mutations present in codons 12, 13, and 61 of the N-*ras* gene and in codons 12 and 61 of the Ki-*ras* and Ha-*ras* genes, we used synthetic 20-mer oligonucleotide probes (Table 1). With these probes, we are able to detect all possible single base pair substitutions in these codons, because a fully matched hybrid between the oligomers and genomic DNA is thermally more stable than a single base pair-mismatched hybrid. Our analysis was conducted as follows: AML DNA was digested with a restriction enzyme (*Pst*I or *Sst*I) and electrophoresed through agarose to separate the restriction fragments. This DNA was hybridized to various ^{32}P-labeled oligomers probes complementary to the sequences that might contain a mutation. The hybridization conditions allow only hybridization of perfectly complementary oligomers and not of those with one or more mismatches. For instance, the N12 oligomer probe (Table 1) is complementary to the sequence around codon 12 of the N-*ras* gene. Under the experimental conditions, this probe will hybridize to the N-*ras* gene only when no mutations are present in codon 12 or codon 13. To detect mutations in the *ras* genes, we prepared a set of probes in which all mutations are represented that can occur in codon 12 and 61 of the Ha-*ras*, Ki-*ras*, and N-*ras* genes and in codon 13 of the N-*ras* gene. Only these codons are affected in naturally occurring tumors. To reduce the number of hybridizations, we used pooled mutation-specific probes. Each pool contains the oligomers homologous to all possible non-silent mutations of one particular base pair. For instance, the N61-p2 oligomer probe (Table 1) consists of three different oligomers homologous to the possible mutations at the second position (p2) of codon 61 of the N-*ras* gene (N61). Figure 1 shows six examples (a through f) of our hybridization analysis. In each example, three different AML DNAs were hybridized to codon 12 probes for either the N-*ras* gene (a through d) or the Ki-*ras* gene (e, f) (lefthand column), and to codon 61 probes for either the N-*ras* gene or the Ki-*ras* gene (middle column). The righthand column represents hybridization with various pools of mutation-specific oligomers that specify the mutation present in the AML DNAs. Among 37 cases of untreated AML, we found seven samples with a *ras* gene mutation (Fig 1): three at position 2 and one at position 3 of codon 61 of the N-*ras* gene, one at position 2 of codon 13 of the N-*ras* gene, and two at position 2 of codon 12 of the Ki-*ras* gene. No mutations were found in codons 12 and 61 of the Ha-*ras* gene or in codon 61 of the Ki-*ras* gene. Subsequently, we also determined the exact base pair change in these samples using individual oligomer probes. The results are shown in Table 2 together with those obtained for the eight AML samples analyzed previously.[8] From them, we conclude that in AML the N-*ras* gene is the preferentially activated member of the *ras* gene family.

Table 1. Sequences of the 20-mer Probes of the N-*ras*, Ki-*ras*, and Ha-*ras* gene

Oligomer	Sequence		Strand*
N12	GGAGCAggtGGTGTTGGGAA		y
N12-p1	------ngt-----------	n = C,A,T	y
N12-p2	------gnt-----------	n = C,A,T	y
N13-p1	---------ngt--------	n = C,A,T	y
N13-p2	---------gnt--------	n = C,A,T	y
N61	TACTCTTCttgTCCAGCTGT		n
N61-p1	--------ttn---------	n = C,A,T	n
N61-p2	--------tng---------	n = C,A,G	n
N61-p3	--------ntg---------	n = A,G,	n
K12	CCTACGCCaccAGCTCCAAC		n
K12-p1	--------acn---------	n = A,T,G	n
K12-p2	--------anc---------	n = A,T,G	n
K61	TACTCCTCttgACCTGCTGT		n
K61-p1	--------ttn---------	n = C,A,T	n
K61-p2	--------tng---------	n = C,A,G	n
K61-p3	--------ntg---------	n = A,G	n
H12	GTGGGCGCCggcGGTGTGGG		y
H12-p1	---------ngc--------	n = C,A,T	y
H12-p2	---------gnc--------	n = C,A,T	y
H61	TACTCCTCctgGCCGGCGGT		n
H61-p1	--------ctn---------	n = C,A,T	n
H61-p2	--------cng---------	n = C,A,G	n
H61-p2	--------ntg---------	n = A,G,	n

Indicated are the oligomers complementary to the normal sequence. Mutation-specific oligomers are derived from these normal oligomers. For instance, N61-p2 is a mixture of oligomers identical to the wt-oligomer except for the normal G-residue at position two of the codon 61. At this position, A, C, and T residues are present in the three oligomers of this mixture.

*Sequence complementary to the coding (n) or noncoding (y) strand.

A *ras* gene can be activated alternatively by amplification of the gene.[15-17] To identify possible amplifications, we hybridized the DNAs with oligomer probes complementary to normal *ras* sequences (Table 1) and compared the hybridization intensities of the wt-probes for the three *ras* oncogenes. In no case was amplification of any of the *ras* genes found (not shown). In all samples in which a mutation was detected, however, both a normal allele and a mutant allele existed. That our samples were consistent for >90% of tumor cells (as determined by light microscopy) suggests that the *ras* gene mutation is heterozygous. Alternatively, the *ras* gene mutation occurs in only a subpopulation of the leukemia cells.

Table 2 includes the classification of the AMLs that contain a *ras* gene mutation. We have not yet found any correlation between the presence of a *ras* gene mutation and cytological features of the leukemia.

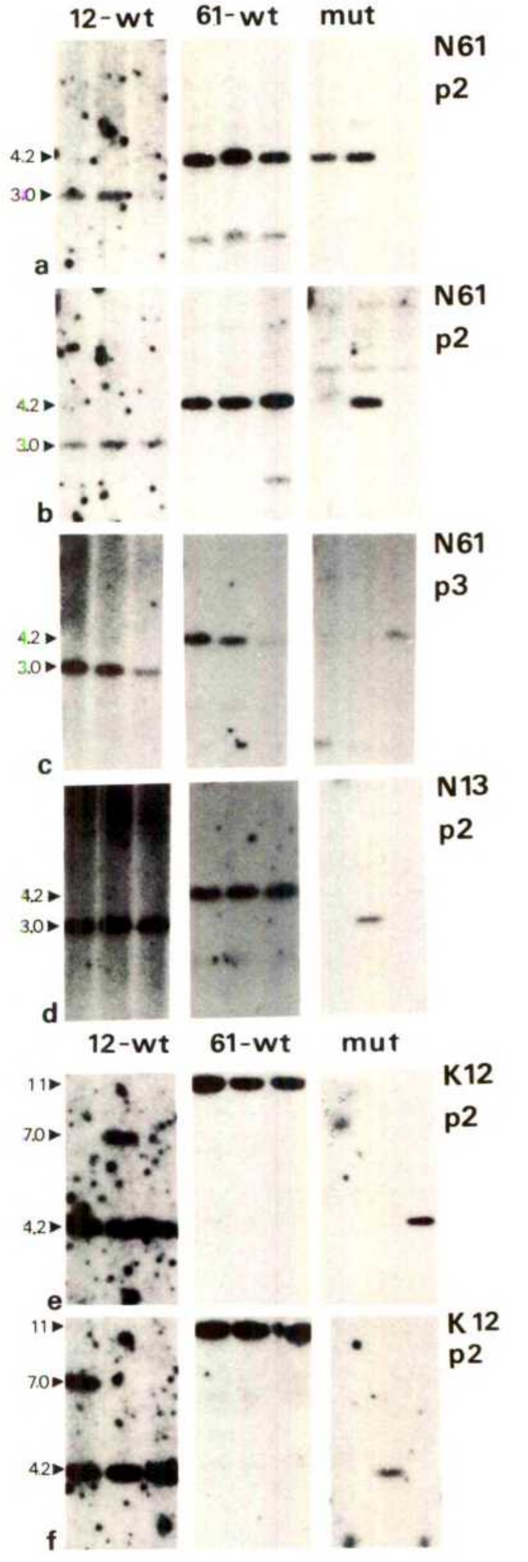

Fig 1. Hybridization of synthetic oligomers to genomic DNA of various samples of acute myeloid leukemia (AML). The DNA was digested with *Pst*I, electrophoresed through agarose, and hybridized in situ to oligomer probes (described in Materials and Methods section): (a) hybridization of three different AML DNAs with the N12-wt oligomer probe (lefthand panel), with the N61-wt oligomer probe (middle panel) and with the mutation-specific oligomer probes N61-p2 (righthand panel); (b through d) as in a, but now in each case three other AML DNAs hybridized with the N12-wt probe, the N61 wt probe, and the mutation-specific probes N61-p2, (b), N61-p3 (c), and N13-p2 (d); (e, f) as in a through d but now Ki-*ras*-specific probes were used: K12-wt (lefthand panel), K61-wt (middle panel), and K12-p2 (righthand panel). The length of the hybridizing fragments is expressed in kilobase pairs (kb). N12 probes hybridize to 3.0-kb *Pst*I fragments, N61 probes to 4.2 kb *Pst*I fragments, and K12 and K61 probes to 4.2-kb and 11-kb *Pst*I fragments, respectively. The 7.0-kb band that hybridizes with the K12-wt probe is a *Pst*I restriction fragment-length polymorphism of the Ki-*ras* gene.

Table 2. *Ras* Gene Mutations in Acute Myeloid Leukemia

	AML	FAB Classification	Mutation				
1	49	M1	N13	GGT	to	GTT	(Val)
2	77	M2	N13	GGT		GTT	(Val)
3	1	M4	N13	GGT		GAT	(Asp)
4	33	M5	N13	GGT		GAT	(Asp)
5	73	M4	N13	GGT		GTT	(Val)
6	MV26	M1	N61	CAA		CGA	(Arg)
7	MV71	M5	K12	GGT		GTT	(Val)
8	MV81	M1	N61	CAA		CGA	(Arg)
9	MV34	M4	N61	CAA		CAN	(His)
10	100	M1	N13	GGT		GTT	(Val)
11	MV65	M4	N61	CAA		CGA	(Arg)
12	MV41	M4	K12	GGT		GAT	(Asp)

AML, acute myeloid leukemia; FAB, French-American-British.

DISCUSSION

Our results and those of others[7] show that *ras* gene mutations occur relatively infrequently, ranging from 5% in mammary tumors[18] and urinary tract tumors[19] to ~25% in AML. In all cases studied, the mutation is specific for the tumor cells and not present in the normal cells of the same individual. The mutations seem to occur randomly among the subtypes of particular malignancies. This may indicate that *ras* gene mutations do not contribute to the genesis of a tumor but arise randomly due to genetic instability of the malignant cells and/or the presence of mutational hot spots at position 12 and 61. In this case, *ras* mutations would be neither essential nor important. Obviously, the latter idea is unlikely, since mutated *ras* oncogenes contribute to the process of carcinogenesis in DNA transfection experiments.[7] A strong indication in support of the importance of *ras*-gene activation in tumorigenesis is the observation that in rats nearly all mammary tumors induced by a single injection of methylnitrosamine contain activated Ha-*ras* genes with a mutation at the same position.[20] An important role of *ras* gene activation in carcinogenesis is further indicated by other studies on chemically induced tumors.[21,22]

A puzzling result is that 5 of the 8 AMLs initially analyzed contained a mutation in codon 13 of the N-*ras* gene,[8] whereas of 37 samples tested later only 1 contained a mutation in that codon, although 6 additional mutations were found at other positions. The high frequency of *ras* mutations in the first eight samples may be explained by the fact that they were analyzed for their ability to oncogenically transform NIH-3T3 cells, as measured by tumor formation in athymic nude mice. This biological assay may be more sensitive than our procedure, especially when only a fraction of the cells in the tumor sample contain a mutation in one of their *ras* genes. This did not seem the case for the first five N-*ras*-13 mutations, however, since they were readily detected by the oligonucleotide probes[8] in the original tumor material, suggesting that the bias for N-*ras*-13 mutations probably is not due to an insufficient sensitivity of our procedure. Moreover, the new *Hph*I site indicative for one of the mutations in the N-*ras*-13 codon (GAT:asp)[8] was not present in other samples of AML (not shown). Because in both studies the samples of AML were collected randomly, the high frequency of N-*ras*-13 mutations in our original study seems to be fortuitous.

Our results show that in AML the preferentially mutated *ras* gene is N-*ras*. This activated oncogene has also been detected in three other samples of AML cells freshly isolated from patients[23-25] and in the AML-cell lines HL60,[12,24] Rc-2a, and KG1.[26] In addition, in acute lymphoid leukemia (ALL) cells, we also found N-*ras* mutations (two N-*ras*-12 mutations in 19 samples analyzed).[27] Probably, N-*ras*, as opposed to the other *ras* genes, plays a predominant role in the control of proliferation and differentiation of cells in the hematopoietic lineage. Alternatively, the preferential activation of N-*ras* may be due to the exposure or sensitivity of myeloid cells to a particular mutagen.

AML is a malignant disorder in which an early progenitor cell is transformed at some point in its differentiation from pluripotent stem cell to committed granulocyte/monocyte colony-forming cells (GM-CFU).[27] The ability of these cells to proliferate and mature is impaired and is manifested in the accumulation of immature blast cells. The differentiation level of the immature cell that accumulates is the basis of the current (French-American-British) classification of AML (M1 through M6). Nonrandom chromosomal aberrations correlate with certain stages of the morphological classification.[28] This suggests that these chromosomal abnormalities affect functions that are responsible for specific steps in differentiation, such as the production of factors responsible for normal myeloid differentiation or cell surface receptors for such factors. Activation of N-*ras*, however, is apparent among different classes of AML, suggesting that N-*ras* does not play a stage-specific role during differentiation. An attractive possibility is that in leukemogenesis an activated N-*ras* gene cooperates with a defect in the maturation pathway, thus providing the leukemic stem cell with the potential of self-renewal, especially when the leukemia arises from a more differentiated, committed stem cell with limited proliferative potential.

It would be interesting to investigate whether N-*ras* activation already occurs at the level of the pluripotent stem cell and is thus present in other nonleukemic, hematopoietic cells as well, or is restricted only to the leukemic cells or their immediate progenitor.

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Improved Results of Treatment of Adult Acute Lymphoblastic Leukemia

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Stephen J. Forman, and Mark J. Farbstein

We designed a treatment program to improve the outcome for adults with acute lymphoblastic leukemia (ALL). Treatment included a remission-induction phase followed by intensive alternating cycles of non-cross-resistant chemotherapy and prolonged oral maintenance therapy. Eighty-one consecutive previously untreated patients were entered on this study. Ninety-four percent of patients entered complete remission. A Kaplan-Meier analysis predicts that 53% ± 9% (SEM) of patients in remission will remain free of disease at 3 years. Neither age, sex, WBC count, nor immunophenotype had a significant effect on remission duration. This program of intensive cyclical chemotherapy has improved the disease-free survival of patients with adult ALL.

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PROGRESS in the treatment of adult acute lymphoblastic leukemia (ALL) has lagged behind that in the treatment of ALL in children. Using relatively nontoxic remission-induction and continuation therapy, 90% of children will enter complete remission, and 50% to 60% of these remissions will be durable.[1,2] Thus, approximately half of the patients with childhood ALL will be cured of their disease. Adults treated with similar programs, however, have only a 50% complete remission rate, and there are few long-term survivors.[3]

This clinical study was initiated with the hope that a different approach to the treatment of adult ALL would yield improved results. Several factors led us to believe that treatment results could be improved. First, reports of ongoing studies in adult ALL suggested that intensified therapy would increase both the complete remission rate and remission duration.[4,5] Second, use of repeated courses of high doses of alternating non–cross-resistant agents produced improved results in the treatment of acute myelogenous leukemia in both children and adults.[6] Third, encouraging reports on the use of the synergistic combination of teniposide (VM-26) and cytosine arabinoside (Ara-C) suggested that a new tool was available for therapy.[7,8]

We designed this treatment protocol with the intent of achieving substantial early cytoreduction using initial multiagent chemotherapy. After delivering prophylactic treatment to the central nervous system (CNS), we administered repeated courses of intensive systemic chemotherapy using alternating courses of several non–cross-resistant drugs. Eligibility was limited to adults aged <50 years because of the anticipated toxicity of the treatment program. This treatment protocol produced a complete remission rate of 94%. By actuarial analysis, we project that 53% ± 9% (SEM) of patients who entered complete remission will remain free of disease at 3 years.

MATERIALS AND METHODS

Patients. Eighty-one consecutive adults with ALL aged <50 years who were seen either at the University of California at San Francisco, City of Hope National Medical Center, Stanford University Medical Center, or by collaborating private physicians in the Bay Area Leukemia Study Group, entered this study between October 1980 and March 1986. During the first 16 months of the study, patients were entered only from the University of California at San Francisco, but beginning in February 1982, patients were entered from all centers. Patients were either previously untreated or had received <2 weeks of therapy with vincristine and prednisone.

Patients were diagnosed as having ALL if morphology was compatible with that diagnosis, if histochemical stains for both peroxidase and α-napthyl butyrate esterase were negative, and if lymphoid markers could be demonstrated. All patients had $>50\%$ blasts in the bone marrow. Immunophenotyping was performed by indirect immunofluorescent techniques using reagents and methods as previously described.[9] The marker used to establish lymphoid lineage was terminal deoxynucleotidyl transferase (TdT) in 72 patients, common ALL antigen (CALLA) in 5 patients, T cell antigen (Leu 1 and Leu 9) in 3 patients, and B cell antigen (BA-1) in 1 patient. Patients were excluded because of prior therapy, presence of Philadelphia chromosome, age >50 years, or diagnosis of B cell leukemia based on L3 morphology and presence of surface immunoglobulin. Central nervous system disease was defined as the presence of any lymphoblasts in cerebrospinal fluid.

Treatment. All patients gave informed consent according to guidelines approved by each institution's Committee on Human Research. Chemotherapy was given in three phases: a remission-induction phase, an intensive alternating combination chemotherapy phase, and a low-dose oral maintenance phase. Remission was induced with daunorubicin, vincristine, prednisone, and L-asparaginase (Table 1). Patients not entering complete remission after 6 weeks of induction chemotherapy were considered to have failed treatment and were taken off study.

Central nervous system prophylaxis was initiated within 1 week of achievement of complete remission. Eighteen hundred rad of cranial radiation were delivered in ten fractions over 12 to 14 days. Six weekly doses of 12 mg of intrathecal methotrexate were administered concurrently. Patients with CNS involvement at diagnosis began their weekly intrathecal methotrexate during induction chemotherapy and received 10 weekly doses. Thereafter, these high-risk patients received intrathecal methotrexate monthly during

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Table 1. Remission Induction Therapy

Daunorubicin	50 mg/m^2 IV, days 1-3
Vincristine	2 mg IV, days 1,8,15,22
Prednisone	60 mg/m^2 PO, days 1-28
L-Asparaginase	6,000 U/m^2 IM, days 17-28
If bone marrow on day 14 has residual leukemia:	
Daunorubicin	50 mg/m^2, day 15
If bone marrow on day 28 has residual leukemia:	
Daunorubicin	50 mg/m^2 IV, days 29,30
Vincristine	2 mg IV, days 29,36
Prednisone	60 mg/m^2 PO, days 29-42
L-Asparaginase	6,000 U/m^2 IM, days 29-35

IV, intravenously; PO, orally; IM, intramuscularly.

the first year of therapy, and the dose of cranial radiation was increased to 2,800 rad.

Courses 1, 3, 5, and 7 of consolidation chemotherapy consisted of daunorubicin, vincristine, prednisone, and L-asparaginase (Table 2). Courses 2, 4, 6, and 8 of consolidation chemotherapy consisted of VM-26 and Ara-C. Courses of consolidation chemotherapy were given approximately monthly after the neutrophil count had risen to >1,000/μL and the platelet count to >100,000/μL. The ninth consolidation course consisted of moderate-dose methotrexate and leucovorin rescue.

Maintenance chemotherapy consisted of methotrexate 20 mg/m^2 orally weekly and 6-mercaptopurine 75 mg/m^2 orally daily. Oral maintenance therapy was continued until 30 months of continuous complete remission; all therapy was then discontinued.

Criteria for response. Patients were considered to be in complete remission when the neutrophil count was >1,500/μL, platelet count was >150,000/μL, and results of bone marrow examination were normal. In addition, patients had to be afebrile and have a normal performance status. Mediastinal masses, peripheral lymphadenopathy, and other extramedullary disease had to have completely resolved.

Supportive care. During remission-induction therapy, patients received prophylactic oral nystatin or clotrimazole, as well as trimethoprim-sulfamethoxasole. The trimethoprim-sulfamethoxasole was continued until 30 months of continuous complete remission. Episodes of fever and neutropenia were treated with broad-spectrum antibiotics, usually an aminoglycoside and a semisynthetic penicillin. Platelet transfusions were given prophylactically to maintain the platelet count >10,000/μL, and RBC transfusions were given according to standard transfusion practice. Granulocyte transfusions were not used.

Table 2. Consolidation Therapy

Treatment A (cycles 1,3,5,7)	
Daunorubicin	50 mg/m^2 IV, days 1,2
Vincristine	2 mg IV, days 1,8
Prednisone	60 mg/m^2 PO days 1-14
L-Asparaginase	12,000 U/m^2 IM days 2,4,7,9,11,14
Treatment B (cycles 2,4,6,8)	
Teniposide	165 mg/m^2 IV, days 1,4,8,11
Cytosine arabinoside	300 mg/m^2 IV, days 1,4,8,11
Treatment C (cycle 9)	
Methotrexate	690 mg/m^2 IV, over 42 hours
Leucovorin	15 mg/m^2 every 6 h × 12 doses beginning at 42 hours

Abbreviations as in Table 1.

Statistical analysis. The duration of remission was measured from the time of the remission bone marrow examination. Survival and disease-free survival were measured from the time of initial therapy. Kaplan-Meier analyses of survival and remission duration were calculated with 95% confidence intervals twice the SEM. Statistical tests on prognostic variables for remission duration were performed with the Mantel Cox test.[10] Deaths in remission are treated as relapses, and withdrawals are evaluated up to the time of withdrawal from the study. Data are analyzed as of July 1986.

RESULTS

Patients. The 81 patients ranged in age from 16 to 48 years (median age 24 years) (Table 3). There were 47 male and 34 female patients. No patients were excluded because of their clinical condition.

Included in the study group are several patients with "unfavorable prognostic features," including 7 patients with initial CNS involvement, 14 with mediastinal masses, 12 with WBC counts >100,000/μL, and 6 with WBC counts >300,000/μL. The presence of a mediastinal mass was strongly associated with male sex (13 of 14), high WBC count (median 69,000/μL), and T cell phenotype (11 of 13 tested). In contrast, CNS involvement was not associated with these features; all seven patients had WBC counts <50,000/μL, and only one had the T cell phenotype.

All patients had the diagnosis of ALL confirmed by the presence of at least one immunologic lymphoid marker. Seventy-two of 75 patients (96%) tested were positive for TdT (Table 4). One TdT-negative patient strongly expressed BA-1, and two patients expressed numerous T cell antigens, including Leu 1 and Leu 9. Sixty-four patients (79% of the study group) were extensively immunophenotyped using monoclonal antibodies. Forty of these 63% were CALLA-positive. Three of these patients had anomalous expression of Leu 5 with no expression of the pan-T antigens Leu 1 or Leu 9. These three patients strongly expressed B cell markers (BA-1 or Leu 12) and are classified as B lineage, CALLA-positive. Four other patients expressed B lineage markers (BA-1 or Leu 12) but were CALLA-negative. Five patients are considered to have "null" cell ALL based on the absence of CALLA, B, or T lineage markers. Fifteen patients had T

Table 3. Clinical Characteristics of Patients

Factor	n
Patients entered	81
Age (yr)	16-48 (median 24)
Sex	
M	47 (58%)
F	34 (42%)
BC count (10^3/μL)	0.2-960 (median 14)
<20,000	47
>20,000	34
<100,000	12
>300,000	6
Mediastinal mass	14 (21%)
CNS disease	7 (9%)

CNS, central nervous system.

Table 4. Immunologic Characteristics of Patients

Phenotype	n	CALLA	B Lineage	E Rosette
Common ALL	40	+	±	±
B lineage	4	−	+	−
Null	5	−	−	−
T	15	±	−	±
Not typed	17	ND	ND	ND

ALL, acute lymphoblastic leukemia; CALLA, common ALL antigen; ND, not determined.

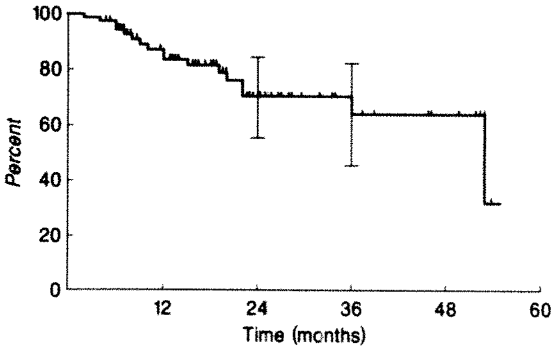

Fig 2. Kaplan-Meier analysis of survival for all patients entered on study. Bars at 24 and 36 months: 95% confidence limits. Tick marks: patients continuing in remission.

cell ALL, based on the presence of Leu 1 or Leu 9. Thirteen were E rosette positive (either by rosette assay or presence of Leu 5), and one of these patients was also CALLA-positive. The E rosette-negative T cell patients expressed Leu 1 and Leu 9, respectively. Two of 20 patients tested expressed the myeloid antigens My7 or My9.

Induction of remission. Seventy-six of 81 patients (94%) achieved complete remission. Only one patient failed to achieve hematologic remission. This patient had progressive disease during the second week of chemotherapy and was taken off study. One patient died of asparaginase hepatotoxicity. Three other patients achieved hematologic remission but had persistent systemic candidiasis that precluded their receiving further chemotherapy; they were therefore considered induction failures. Of 76 patients in remission, 41 received 3 doses of daunorubicin, 31 required 4 doses of daunorubicin, and 4 patients had therapy extended to 6 weeks before achieving complete remission.

Duration of remission and survival. Kaplan-Meier analysis projects that 53% ± 9% (SEM) of patients achieving complete remission will remain in continuous complete remission at 3 years (Fig 1). Kaplan-Meier analysis projects that 64% ± 9% (SEM) of all patients will be alive at 3 years (Fig 2). Disease-free survival for all patients entered on study is projected to be 50% ± 9% (SEM) at 3 years (Fig 3).

Of 76 patients in complete remission, 15 have been withdrawn from study, 3 have died in complete remission, and 18 have relapsed (Table 5). Forty patients remain in continuous complete remission (CCR) from 3 to 52 months, with median follow-up of 16 months. Twenty-seven patients have been in remission >1 year, 17 patients have been in remission 2 years, and 8 patients remain in remission >3 years.

Fifteen patients were withdrawn from study. These patients did not have a high incidence of unfavorable prognostic features and were not selected for alternative therapy because of an anticipated poor outcome. Six were treated with bone marrow transplantation, 1 developed anaphylaxis to the initial dose of VM-26, 5 moved or were lost to follow-up, and 3 were withdrawn because of major protocol violations. Two of these violations occurred because the patient refused further therapy; one violation occurred because the physician refused to administer scheduled therapy (despite adequate peripheral blood counts) because of marked bone marrow hypocellularity.

There have been 18 relapses between 1 and 51 months of complete remission. Seventeen relapses occurred in bone marrow. One relapse (in a patient with CNS disease at initial diagnosis) occurred simultaneously in the bone marrow and CNS. Most relapses occurred early, and only three patients relapsed after 19 months. The late relapse at 51 months developed in a patient with transfusion-related AIDS.

Factors that may have influenced remission duration were analyzed (Table 6). Neither age, sex, WBC count, presence of CNS disease, number of doses of daunorubicin required to induce remission, nor immunologic subtype had a significant effect on remission duration (Fig 4).

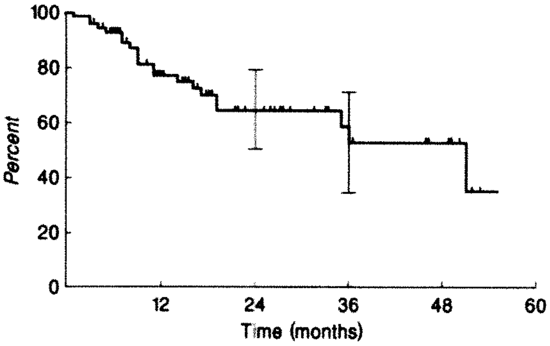

Fig 1. Kaplan-Meier analysis of remission duration for patients in remission. Bars at 24 and 36 months: 95% confidence limits. Tick marks: patients continuing in remission.

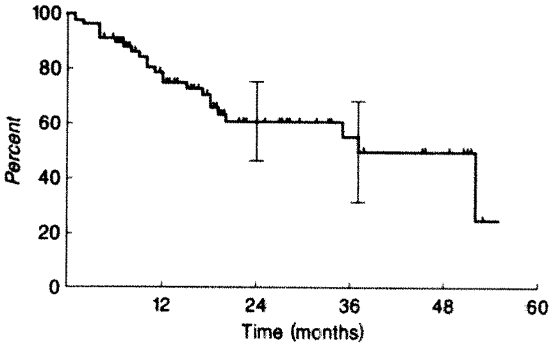

Fig 3. Kaplan-Meier analysis of disease-free survival for all patients entered on study. Bars at 24 and 36 months: 95% confidence limits. Tick marks: patients continuing in remission.

Table 5. Results of Therapy

Result	n
Patients entered	81
Chemotherapy failure	1
Death during induction	1
Other induction failure	3
Complete remission	76
Withdrawal	15
Death in remission	3
Relapse	18
Continuous remission	40

Table 6. Effect of Prognostic Variables on Remission Duration

	n (CR)	n (Not withdrawn)	2-year CCR (% ± SEM)
All patients	76	61	65 ± 7
Age (yr)			
<20	17	13	56 ± 15
>30	21	19	59 ± 16
Sex			
M	46	36	63 ± 9
F	30	25	68 ± 11
WBC count ($10^3/\mu L$)			
<10,000	31	25	66 ± 11
<20,000	47	39	63 ± 9
>50,000	19	16	74 ± 11
>100,000	12	10	60 ± 18
Phenotype			
Calla	38	33	59 ± 10
T	15	12	61 ± 17
Doses daunorubicin to CR			
3	41	32	63 ± 10
4	31	25	65 ± 11
6	4	4	75 ± 22
CNS disease			
No	70	55	63 ± 8
Yes	6	6	80 ± 18

CNS, central nervous system; CR, complete remission; CCR, continuous complete remission.

Toxicity. During remission-induction therapy, all patients had reversible alopecia. Nausea and vomiting were minimal, and stomatitis did not occur. Peripheral neuropathy related to vincristine was usually mild and was significant only in the small group of patients who received 6 weeks of induction chemotherapy.

Side effects related to L-asparaginase were common but were rarely threatening. One patient died during induction therapy of asparaginase hepatotoxicity. One patient developed severe hypersensitivity to the initial dose of *Escherichia coli* asparaginase and was treated with *Erwinia* asparaginase without problems. One patient developed symptomatic pancreatitis. One patient developed severe hepatic and renal toxicity attributed to asparaginase after 7 doses and another received only 10 of 12 planned doses because of progressive lethargy. Subclavian thromboses at the site of the Hickman catheter occurred in three patients. Abnormalities of glucose tolerance and liver function were common but were not considered an indication to discontinue the drug.

Hematologic toxicity of remission-induction therapy was within the expected range. Thrombocytopenia developed in most patients, and 70% required platelet transfusions. The platelets recovered to $>100{,}000/\mu L$ at a median of 18 days. Neutropenia developed in all patients, and 62% were treated with systemic antibiotics other than prophylactic low-dose trimethroprimsulfamethoxasole. There were no infection-related deaths, but three patients failed to respond adequately to amphotericin treatment of systemic candidiasis and could not receive planned consolidation chemotherapy. The median duration of hospitalization for patients during remission-induction was 28 days.

Central nervous system prophylaxis with cranial radiation and intrathecal methotrexate was generally well tolerated. Symptoms of arachnoiditis with headache, nausea, and vomiting developed in 20% of patients, and delayed toxicity of cranial radiation (somnolence syndrome) occurred in 10% of patients.

Consolidation therapy with daunorubicin, vincristine, prednisone, and asparaginase was complicated by fever requiring antibiotics in 10% (21 of 219) of courses. Patients often developed profound neutropenia ($<100/\mu L$), but the duration of severe neutropenia was brief. The median length of these cycles of therapy until the next chemotherapy was 33 days. Several complications of L-asparaginase treatment occurred during these consolidation cycles. One patient developed severe hemorrhagic pancreatitis. She recovered from this episode but died during her next chemotherapy course of noninfectious respiratory failure associated with numerous pancreatic pseudocysts. One patient developed severe hypersensitivity and was subsequently treated with *Erwinia* asparaginase without adverse events. One patient developed massive femoral and iliac venous thrombosis and was successfully treated with streptokinase and heparin. Three patients developed clinical evidence of cardiomyopathy. One had inadvertently received three doses of doxorubicin (50 mg/m^2) during induction chemotherapy and had subsequently received a cumulative dose of 400 mg/m^2 of daunorubicin during consolidation therapy. Two others had received cumulative doses of 600 mg/m^2 of daunorubicin.

Consolidation therapy with VM-26 plus Ara-C was complicated by fever and neutropenia requiring systemic antibi-

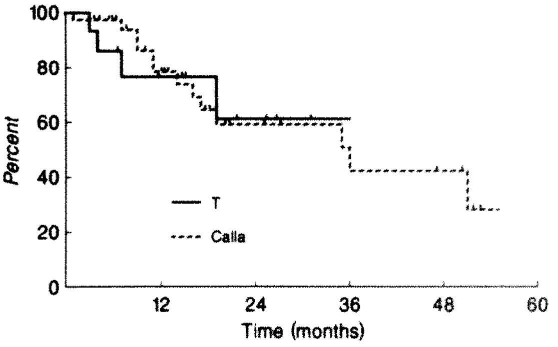

Fig 4. Kaplan-Meier analysis of remission duration by immunophenotype. Tick marks: patients continuing in remission.

otics in 39% (74 of 191) of courses. Profound neutropenia developed in most patients and usually lasted 7 to 10 days. Infections were most commonly caused by enteric gram-negative organisms, but several patients developed *Candida* esophagitis and one patient developed disseminated candidiasis. There were 2 treatment-related deaths, 1 due to noninfectious respiratory failure associated with pancreatic pseudocysts, and 1 due to marantic endocarditis superimposed on underlying rheumatic valvular disease.

The final consolidation therapy treatment with methotrexate was well tolerated. Mild mucositis occurred infrequently, and there was no significant hematologic toxicity. Oral maintenance therapy was generally well tolerated. However, the majority of the patients received 75% of the planned dose because of hematologic toxicity, and one patient with cardiomyopathy died of infection.

Median hospitalization time during the entirety of post-remission-induction therapy was 19 days. Four of these days were required for administration of the methotrexate infusion, and 15 days were required for antibiotic treatment of episodes of fever and neutropenia. Patients thus spent a median of 47 days in the hospital during the entire period of treatment.

DISCUSSION

The question of the criteria for the diagnosis of adult ALL is a difficult one. The results of the treatment program may be critically dependent on the population studied. The diagnosis of ALL is not as straightforward in adults as it is in children, since the typical L1 morphology is apparent in only 20% to 30% of cases. We used available diagnostic techniques to define our ALL population as accurately as possible. We required not only that cells fail to exhibit myeloid characteristics by histochemical stains, but also that the cells be positively identified as lymphoid by an immunologic technique. This was done in an attempt to exclude occult cases of acute myeloid leukemia that would exhibit peroxidase positivity only at an ultrastructural level.[11]

Our patient group is representative of most groups of adult ALL. No patients were excluded because of their clinical condition. Included in our treatment group are several patients traditionally defined as having poor prognostic features. Seven patients initially had CNS involvement, 12 patients had WBC counts $>100{,}000/\mu L$, and 6 patients had extreme hyperleucocytosis with WBC counts $>300{,}000/\mu L$. Seventy-nine percent of patients were extensively immunophenotyped using monoclonal antibodies. The most common reason for failure to phenotype was inaspirable bone marrow with low circulating blast counts. We were able to phenotype some of these patients, however, using in vitro antibody binding to biopsies placed in saline.[12] The distribution of immunologic subtypes of ALL in our study is similar to that in other reported series (Table 4). Sixty-three percent of patients studied were classified as "common" ALL, and 23% had T cell disease.

The goal of our induction chemotherapy was to achieve normal bone marrow function (complete remission) as quickly as possible without undue toxicity. The importance of avoiding toxicity is highlighted by the fact that, in the past, more induction failures had been due to therapy-related complications than to chemotherapy-resistant disease.[13-15] On the other hand, early studies at St Jude's Hospital suggested that the results of the early phases of treatment were critical in determining overall outcome and that more intensive subsequent treatment may not be able to overcome poor initial cytoreduction.[1] Recently, the importance of intensive induction chemotherapy was demonstrated by two groups treating childhood ALL.[16] In one prospective randomized clinical study of childhood ALL, the addition of an anthracycline to the remission-induction program improved long-term disease-free survival from 39% to 64%.

At a minimum, an optimal remission-induction program for adult ALL should include an anthracycline in addition to vincristine and prednisone. The Cancer and Leukemia Group B recently demonstrated in a randomized prospective study that adding daunorubicin to vincristine and prednisone increased the remission rate from 47% to 78%.[17] Although the period of neutropenia was increased by the addition of daunorubicin, no increase in either severe or fatal infections occurred in the more intensively treated group. One may actually be able to reduce overall toxicity by achieving complete remission more rapidly, thereby avoiding prolonged pancytopenia caused by the sequential addition of increments of therapy. Several other recent studies showed that the combination of daunorubicin, vincristine, and prednisone consistently produced a complete remission of ~80% in adult ALL.[18,19]

Given the complete remission rate of 80% using daunorubicin, vincristine, and prednisone, and the relatively small number of patients with adult ALL available for study, one cannot demonstrate a remission-induction advantage to the addition of asparaginase to this program.[20-25] Asparaginase can, however, be a useful agent for remission induction, and the best reported results for remission induction in children with relapsed ALL have been produced by four-drug regimens that included asparaginase.[26,27]

Our remission-induction program has been very successful, with 94% of patients entering complete remission. The toxicity of the program was not severe, and profound neutropenia usually lasted only several days. Systemic antibiotics were needed in only 62% of patients, and life-threatening infections were not common. Patients were generally in excellent clinical condition at the completion of induction therapy and could proceed directly to consolidation therapy without delay.

One novel aspect of our treatment program was the repeated use of VM-26 and Ara-C in consolidation therapy. It was the early experience with this synergistic combination that prompted the design of this study. In early studies, VM-26 produced a low response rate when used as a single agent. Increasing the dose and adding vincristine and prednisone produced a 30% remission rate in children with relapsed ALL, however.[28] VM-26 given in combination with Ara-C, produced exciting results.[7,8] In one study, 3 of 4 patients who failed to respond to initial intensive induction therapy entered complete remission, and 2 of these patients remained in remission 30 months later when they completed therapy. To have produced this disease-free survival in patients with a dismal prognosis was unprecedented, and suggested that this combination would be a major addition to our therapeutic armamentarium. Further studies suggested a beneficial role for VM-26 plus Ara-C as consolidation therapy for ALL. One report demonstrated that the addition of a single consolidation course with these agents prolonged remission duration and produced more long-term disease-free survivors.[29] This, however, was not confirmed in a second study.[30] Repeated courses of VM-26 plus Ara-C have improved the outcome in high-risk pediatric ALL.[31]

Alternating with cycles of VM-26 plus Ara-C, we treated patients with a four-drug program that included asparaginase in a total dose of 72,000 U/m^2. We chose this dose of asparaginase based on dose–response relationships established in pediatric ALL.[32,33] Few adult treatment programs have used repeated large doses of L-asparaginase,[34] and this represents a second novel aspect of our treatment program. The potential usefulness of asparaginase in consolidation therapy in children has been demonstrated by a randomized study showing longer disease-free survival in asparaginase-treated patients.[35]

One of the goals of the study was to determine if more effective therapy would change the significance of factors previously reported to be of prognostic importance in adult ALL. We particularly wondered if more effective therapy would diminish the importance of adverse prognostic features. Most reported studies of adult ALL show a strong prognostic significance for WBC count at diagnosis.[13,18,20,21,25] The prognostic significance of immunologic subtype has been variable.[13,18,20,21,36] At this point in our analysis, neither age, sex, WBC count, nor immunologic subtype has prognostic significance with respect to either remission induction or remission duration. Our assessment of prognostic factors differs from those of previous studies. Because many of our patients are still at risk for relapse, however, it is too early to draw firm conclusions from this analysis.

Intensified chemotherapy has been convincingly shown to improve the outcome in high-risk pediatric ALL.[37] Our treatment results are very encouraging and add to the increasing evidence that intensive treatment has improved the long-term outcome of adult ALL as well. During the 1970s, only 15% of adult ALL patients remained disease-free at 5 years. The L10 protocol at Memorial Hospital now has a large enough patient group and long enough follow-up to be confident that results of treatment have indeed improved.[13,18] Thirty-five percent to 40% of all patients entered are projected to remain disease-free at 5 years, and most are probably being cured of their disease.

In conclusion, we have developed an intensive treatment program for adult ALL that has been successful in improving disease-free survival. Although our treatment program is prolonged, the intensive phase is completed in 11 months and subsequent oral maintenance therapy is very well tolerated. The program was carried out at several different institutions as well as by private physicians and was relatively easy to administer. Patients spent <7 weeks in the hospital during all therapy. Although longer follow-up will be necessary to determine the ultimate impact of this program, we are hopeful that a substantial fraction of patients will be cured of their disease.

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In Vitro Tests That Predict Tumor-Associated Idiotype Levels in the Serum of Patients With B Cell Lymphomas and Leukemias

By Richard A. Miller, James N. Lowder, Julie Gralow, Timothy Meeker, and Ronald Levy

The presence of circulating tumor idiotype interferes with the in vivo effectiveness of anti-idiotype antibodies. We developed two assays that permit identification of patients with high levels of serum idiotype without the need for first producing an anti-idiotype antibody. A cell suspension made from the tumor was cultured for seven days with or without phytohemagglutinin (PHA) and/or phorbol myristic acetate (PMA). Ig secretion in vitro by patients' tumor cells varied. In 4 patients, no secretion in vitro occurred, 5 patients had low levels, and 5 patients had high levels of Ig secretion. In three patients, Ig secretion occurred only after stimulation with PHA, PMA, or both. Spontaneous or induced immunoglobulin secretion in vitro is related to the levels of tumor idiotype secretion that exist in vivo. Eight patients with serum idiotype levels $>100\ \mu g/mL$ (mean 265 $\mu g/mL$), had a minimum of 1.0 $\mu g/10^6$ cells of idiotype secretion in vitro. Nine patients with serum idiotype levels $<30\ \mu g/mL$ (mean 3.7 $\mu g/mL$), had $\leq 0.5\ \mu g/10^6$ cells of idiotype secretion in vitro. In another assay, the levels of IgM κ and IgM λ in patients' sera were compared with those in normal serum. An imbalance in the relative amounts of IgM κ and IgM λ indicated high levels of circulating idiotype in the serum, but this assay was less sensitive than the in vitro secretion assay and limited to IgM-secreting tumors. These assays will be useful for future clinical studies using anti-idiotype antibodies.

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MOST MALIGNANT lymphoproliferative diseases that occur in humans are derived from B lymphocytes and synthesize an immunoglobulin (Ig) with a unique combination of a V_L and V_H region, and a light chain of either the κ or λ type.[1,2] Antigenic determinants unique to these Igs are called idiotypes and represent a specific marker for that tumor. In some diseases, eg, multiple myeloma and Waldenstrom's macroglobulinemia, the malignant cells secrete large amounts of Ig. In most cases, however, the Ig is expressed on the cell surface without significant secretion.[3] We and others prepared antibodies against tumor idiotypes and showed that such reagents are useful for studies of the immunobiology of these diseases.[4-9] Antiidiotype antibodies can also be used to monitor tumor growth in humans and in experimental animals.[10-12] For several reasons, anti-idiotype antibodies offer promise as therapeutic agents. Animal studies and human therapeutic trials have demonstrated the potential for this approach.[4,6,8,9,13,14] Our own therapeutic trials in 14 patients with B cell lymphomas using monoclonal anti-idiotype antibodies demonstrated variable results.[9,15] In one case, a complete and durable remission was achieved. In other cases, partial responses occurred.[8,9] In these studies, the presence of circulating tumor idiotype interfered with the therapeutic use of anti-idiotype antibody. In addition to blocking the anti-idiotype antibody from binding to cellular antigen, circulating idiotype–anti-idiotype complexes led to toxicity.

To avoid these problems, one should select patients with little or no circulating idiotype. Conventional serologic techniques, such as serum protein electrophoresis or quantitative Ig levels, will exclude the minority of patients with very high levels of paraprotein but are not sensitive enough to detect levels of serum idiotype $<200\ \mu g/mL$, which are capable of interfering with anti-idiotype therapy.

We developed in vitro techniques that allow prediction of the level of tumor idiotype in a patient's serum. These techniques are particularly valuable because they do not require availability of an anti-idiotype antibody.

MATERIALS AND METHODS

Patients and preparation of tissues. Eighteen patients with established histopathologic diagnoses of B cell malignancies were included in these studies (Table 1). Patients were selected who had tumor tissue readily accessible to biopsy and expected survivals of >1 year. Cell suspensions were made either from lymph nodes obtained by biopsy or from spleens removed during laparotomy. In patients with leukemia, peripheral blood was used as the source of tumor cells. After isolation of mononuclear cells using Ficoll-Hypaque centrifugation, cells were suspended in medium containing 10% dimethyl sulfoxide (DMSO) and cryopreserved in liquid nitrogen. Immunophenotyping was performed either on fresh cells or on cells that had been stored in liquid nitrogen. Monoclonal fluorescein conjugated anti-Leu-4 (Becton Dickinson Monoclonal Center, Mountain View, CA) was used to identify normal mature T cells. Affinity-purified fluorescein-conjugated $F(ab)_2$ fragments of goat anti-human Ig heavy and light chains (Tago, Burlingame, CA) were used to identify normal and malignant B cells. Immunofluorescence staining and analysis using a fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Sunnyvale, CA) were described previously.[9,16] At the time of biopsy, patients' sera were obtained by venipuncture, separated from clotted whole blood by centrifugation at 1,000 g, and stored at −20°C.

Spontaneous or induced secretion of Ig in vitro. Patient cells were thawed, and viable mononuclear cells were isolated by Ficoll-Hypaque separation. Viability proved >95% after centrifugation, as measured by trypan blue dye exclusion. One million cells per milliliter were resuspended in RPMI 1640 medium containing 15% fetal calf serum (FCS) (GIBCO, Grand Island, NY), L-glutamine, sodium pyruvate, penicillin, streptomycin, and 10^{-4} mol/L of 2-mercaptoethanol. One milliliter of the patients' cell suspensions was

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Table 1. Characteristics of Patients' Tissues

Patient	Diagnosis*	Tissue Source*	Ig Type	Percentage of Cells† Tumor	T
C.B.	NLPD	Spleen	μ, κ	94	6
R.B.	NLPD	LN	μ, κ	53	45
J.C.	NLPD	Spleen	γ, λ	86	14
K.C.	NLPD	LN	γ, κ	84	16
N.D.	DM	LN	μ, λ	74	22
P.E.	DLPD	LN	μ, κ	21	76
T.G.	NLPD	PBL	μ, γ, λ	93	6
C.G.	NM	Spleen	μ, κ	79	18
D.H.	CLL	PBL	μ, κ μ, λ	86	14
B.J.	NLPD	LN	μ, κ	69	20
C.J.	NLPD	LN	μ, κ	86	14
B.L.	NLPD	LN	μ, κ	84	15
C.P.	NM	LN	μ, λ	73	25
R.P.	CLL	PBL	μ, κ	79	19
L.R.	NLPD	LN	μ, λ	73	24
D.S.	NLPD	LN	γ, λ	85	13
F.S.	PLL	PBL	μ, κ	94	6
M.V.	DLPD	PBL	μ, κ	84	13

*NLPD, nodular lymphoma, lymphocytic, poorly differentiated type (follicular, small cleaved cell); DM, diffuse lymphoma, mixed histiocytic-lymphocytic type (diffuse mixed small cleaved and large cell); DLPD, diffuse lymphoma, lymphocytic, poorly differentiated type (diffuse, small cleaved cell); NM, nodular lymphoma, mixed histiocytic-lymphocytic type (follicular, mixed small cleaved and large cell); CLL, chronic lymphocytic leukemia; PLL, prolymphocytic leukemia; LN, lymph node; PBL, peripheral blood lymphocytes.

†Percentage of tumor cells and T cells determined by immunofluorescence staining and analysis using flow cytometry. Calculation of tumor cells based on staining with either anti-κ or anti-λ reagent. Percentage of T cells was determined using anti-Leu-4.

placed in each of four wells in a 24-well tissue culture plate (Costar, Cambridge, MA). Either 1.0 μg of phytohemagglutinin (PHA, Burroughs Wellcome, Research Triangle, NC) or 10 ng of phorbol myristic acetate (PMA, Sigma, St Louis) or a combination of both at the same final concentrations were added to individual wells. Control and stimulated cultures were maintained in a humidified 37°C, 5% CO_2 atmosphere in an air incubator for seven days. Kinetic studies were first done to determine the optimal incubation time at which to assay for Ig secretion.

Immunoglobulin assay. The amount of Ig in culture supernatants was measured using an enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates (96-well) (Immulon I, Dynatech, Alexandria, VA) were coated with 50 μL/well of affinity-purified goat anti-human Ig (Tago) at a concentration of 10 μg/mL in phosphate-buffered saline (PBS). After the cell cultures were washed with PBS containing 0.05% NP-40 (Sigma), 50 μL of culture supernatants, serially diluted in 1% bovine serum albumin (BSA) in PBS, was added to the wells for one hour at room temperature. The plates were washed again, and 50 μL of horseradish peroxidase (HRP) conjugated, affinity-purified, goat anti-human κ or λ light chain-specific antibodies (Tago) was added for one hour at room temperature. After the cultures were washed, 100 μL of a solution of 2, 2'-azino-di-(3-ethyl-benzthiazoline-sulfonate) (ABTS, Sigma) in 0.05 mol/L of sodium citrate buffer pH 4.0 with 0.01% hydrogen peroxide was added. Color development was determined by measuring the optical density (OD) at 405 nm using an MR600 microplate reader (Dynatech). From the mean OD, the amount of Ig was calculated from a standard curve produced using purified human Igs. Although some assays were done on different days, the amount of Ig was always calculated based on simultaneously run standards. The sensitivity of this assay was 0.01 μg/mL.

Assay for IgM κ and λ light chains. Polystyrene microtiter plates were coated with 10 μg/mL of goat anti-human IgM (Tago) in PBS. The plates were washed as above and then incubated for one hour at room temperature with 5% instant milk (Carnation Nonfat Dry Milk, Los Angeles) in PBS. This blocking step was found to increase the sensitivity of the assay greatly by reducing the background. After another wash, the patients' sera were added by doubling dilutions with PBS containing 1% BSA (PBS–BSA) into 16 wells. The plates were incubated for one hour at room temperature and washed again. Horseradish peroxidase-conjugated affinity-purified goat anti-human κ or λ light chain-specific antibodies (Tago) were added. After one-hour incubation at room temperature and another wash, an ABTS solution was added and color development was measured as described above. This assay was performed simultaneously on the test samples and pooled normal human serum (HS, eight donors). Titration curves were generated, and the 50% saturation points were compared. The serum dilutions at these points were expressed as a percentage of the normal pooled HS. The amounts of IgM κ, relative to pooled normal HS, was then compared with the amount of IgM λ, relative to pooled normal HS.

Anti-idiotype antibodies. Murine monoclonal anti-idiotype antibodies were produced as previously described.[17] Either purified idiotypic protein or whole tumor cells were used to immunize mice used in the making of anti-idiotype antibody-secreting hybridomas. The candidate anti-idiotypes were screened for nonreactivity with three different purified human IgM proteins and human tonsil. The antibodies used in these studies were purified from ascites.

Measurement of tumor-specific serum idiotype. An ELISA assay used to measure the concentration of serum idiotype was described previously.[9] The microtiter plates (Dynatech) were coated with specific anti-idiotype antibody at a concentration of 10 μg/mL in PBS NP-40. After the plates were washed, idiotype protein standard and unknown samples were serially diluted with PBS-BSA in parallel on the plate, incubated, and washed again. Biotin-conjugated specific monoclonal anti-idiotype antibody was added, incubated, and washed from the plate. Avidin conjugated to HRP (Vector, Burlingame, CA) was added at an appropriate dilution, incubated, and washed. ABTS was added as a final step, and the results were evaluated as in the immunoglobulin assay described above.

RESULTS

Characteristics of patients and tissues. Table 1 lists the 18 patients selected for these studies. All patients had advanced disease with easily palpable lymphadenopathy in multiple anatomic sites and/or large numbers of tumor cells in their peripheral blood. Lymphoma patients had stage III or IV disease; leukemic patients had WBC counts >25,000/μL, and most cells were malignant. Eleven of the 18 patients had received prior treatment with chemotherapy and/or radiotherapy. No patient had received therapy within 1 month of tissue sampling or serum collection. The sources of tissue were lymph node in 10 patients, peripheral blood in 5 patients, and spleen in 3 patients who underwent splenectomy for diagnosis (C.B.) or because of cytopenias or symptomatic organomegaly (J.C., C.G.).

The surface Ig type was established by immunofluorescence staining and FACS analysis of cell suspensions. In each case, except that of D.H., a predominant light chain type was found. Thus, these cases were believed to be monoclonal. These results were confirmed on frozen tissue samples using an immunoperoxidase technique.[16] In D.H., the peripheral blood lymphocytes expressed either κ or λ light chain in approximately equal ratios.

The percentage of malignant cells and infiltrating normal T cells within the tissue are also shown in Table 1. These percentages were determined using immunofluorescence on the FACS gated on the lymphoid population of cells. The degree of tumor involvement was >50% in the specimens of 17 of 18 patients. One patient's (P.E.) biopsy had only 21% tumor cells, with a strikingly large number of normal T cells (76%).

Secretion of tumor idiotype in vitro. Cell suspensions from tumor specimens were cultured with or without PMA and/or PHA. The optimal concentration of PMA (10 ng/mL) and time to measure secreted Ig in the culture supernatant (day 7) were determined in preliminary studies. Subsequently, cumulative Ig secretion was measured after a seven-day culture period (Table 2). Four patients (T.G., B.J., C.J., and C.P.) had undetectable amounts of Ig in control and in stimulated cultures. Five patients (R.B., K.C., N.D., C.G., and D.S.) had low levels of spontaneous or induced Ig secretion. The remaining nine patients had higher levels of secreted Ig. Three patients (R.P., L.R., and M.V.) had no detectable spontaneous Ig secretion, but significant quantities were secreted on stimulation with PMA, PHA, or both. Five patients (J.C., P.E., D.H., B.L., and F.S.) had high levels of spontaneous Ig secretion only minimally affected by stimulation.

Table 2. Secretion of Tumor Id In Vitro and In Vivo

	In Vitro Secretion (μg/mL)					
Patient	No Mitogen	PMA	PHA	PMA + PHA	Serum Id Level (μg/mL)	Serum κ-λ Imbalance
C.B.	0.8	0.8	0.8	1.0	100	Yes
R.B.	0.15	0.25	0.15	0.4	0.01	No
J.C.	1.4	0.7	1.4	1.3	>150	NA
K.C.	0.10	0.05	0.10	0.15	30	NA
N.D.	0.2	0.4	0.5	0.5	0.1	No
P.E.	2.0	2.0	2.0	2.0	150	Yes
T.G.	0	0	0	0	15	No
C.G.	0.3	0.4	0.4	0.4	0	No
D.H.*						
(κ)	0.6	1.8	0.6	1.0	NM	NM
(λ)	0.5	1.0	0.5	0.6		
B.J.	0	0	0	0	0.02	No
C.J.	0	0	0	0	2.2	No
B.L.	0.8	1.0	1.0	1.5	120	Yes
C.P.	0	0	0	0	0	No
R.P.	0	0.6	1.5	3.0	<500	Yes
L.R.	0	0.15	0	1.2	>200	Yes
D.S.	0	0.05	0	0.05	<1	NA
F.S.	0.9	0.6	1.1	1.4	500	Yes
M.V.	0	1.0	0.6	1.1	400	Yes

Id, idiotype; PMA, phorbol myristate acetate; PHA, phytohemagglutinin. 0, none detectable; NM, not measured; NA, not applicable because tumor secreted IgG.

*Secretion of both light chain classes was observed. Values for both are listed.

In all but one case, only a single type of secreted light chain was detected. This always matched the light chain type found on the surface of the tumor cells. In D.H., both κ and λ light chains were found in culture supernatants. Immunoglobulin gene rearrangement studies showed that patient D.H. was biclonal as was reported previously.[18] His tumor was comprised of a mixture of κ-and λ-bearing clones.

Correlation of secretion in vitro with serum idiotype level. Table 2 also shows the amount of tumor idiotype measured in the serum of these patients at the time of tissue sampling. The levels of serum idiotype correlated with the spontaneous or induced secretion of Ig in vitro (Fig 1). Patients with spontaneous or induced levels of secreted Ig ≥1.0 μg/mL had high serum idiotype levels (mean ± SD 265 μg/mL ± 172) as compared with patients with secretion in vitro ≤0.5 μg/mL (mean ± SD 3.7 μg/mL ± 9.9). Distribution of serum idiotype levels between these two groups did not overlap. The measurement of spontaneous or induced Ig secretion in vitro accurately identified patients with high serum idiotype levels. There was no correlation between the numbers of normal T cells within the tumor sample and the amount of spontaneous or induced Ig secretion in vitro (Tables 1 and 2). Further analysis of T cell subsets revealed no consistent pattern (data not shown).

Analysis of serum IgM for κ and λ light chain. Most B cell lymphomas express IgM on their cell surface and would secrete antibody with that heavy chain isotype. The total amounts of IgM κ and IgM λ in the serum of most of the patients were below those found in pooled normal serum. This finding is a result of the hypogammaglobulinemia that frequently exists in patients with B cell lymphoma. An assay that measured the concentration of κ and λ light chains in the IgM component of serum was developed. The values were compared with those in pooled normal human serum; a calibration of this assay is shown in Fig 2. Various concentrations of either a purified IgM κ or IgM λ paraprotein were added to pooled human serum and compared with the unspiked serum. A minimum level of paraprotein of 200 μg/mL was detectable in this assay. Higher levels caused an

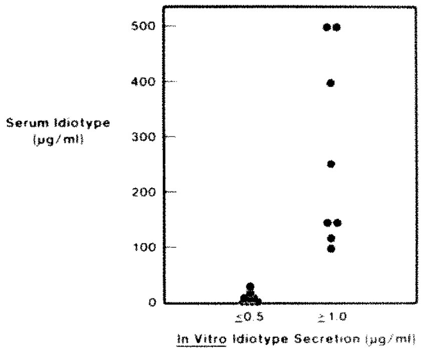

Fig 1. Correlation of in vitro secretion of Ig with serum idiotype level. Tumor cells secreting ≤0.5 μg/mL of Ig in vitro were from patients with ≤30 μg/mL of serum idiotype. In vitro secretion of ≥1.0 μg/mL predicted ≥100 μg/mL of serum idiotype.

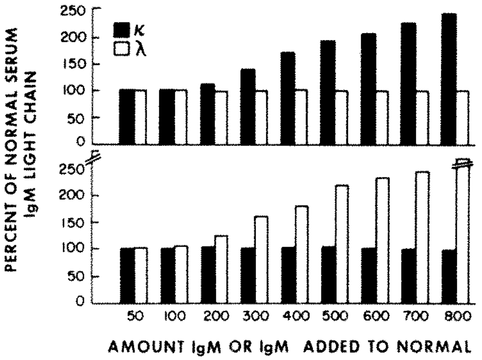

Fig 2. Sensitivity of κ/λ ratio assay for the presence of IgM paraprotein. Increasing amounts of purified $\mu\kappa$ (top) or $\mu\lambda$ (bottom) Ig were added to normal human serum and assayed as described in the Materials and Methods section. SD was <10% both in repeated assays and when different sources of normal human serum were used.

increasingly greater excess of light chain in the test serum as compared with the unspiked serum. As expected, the non-paraprotein–associated light chain levels remained constant in all the test samples. Patients with IgG-secreting tumors were tested using a similar assay. The large amounts of normal IgG in serum obscured the results.

This assay was performed on serum from 14 patients, as shown in Fig 3. Seven patients (C.B., P.E., B.L., R.P., L.R., F.S., and M.V) had an imbalance in the amounts of light chains as compared with patients having normal serum. This is best demonstrated in patient R.P., who had a monoclonal IgM κ protein spike detected by serum protein electrophoresis. Using this assay, we found more IgM κ in the patient's serum than in the normal serum; however, the IgM λ level was quite low. This caused a dramatic imbalance in the levels of IgM light chains as compared with those in normal serum. Similar, but less dramatic, results were obtained in six other patients. Each of these seven patients had high levels (>100 μg/mL) of idiotype in the serum. The other seven patients had no significant imbalance in IgM light chains and had serum idiotype levels <15 μg/mL. As compared with the data shown in Fig 2, detectable levels of circulating monoclonal proteins were lower in the patient's serum (100 μg/ml in C.B.) than in normal serum. This is likely related to the lower background IgM levels, which made it easier to detect small amounts of the monoclonal Ig. This assay successfully predicted which patients secreted $\geq$100 μg/mL of idiotype into the serum, provided that the idiotype was IgM.

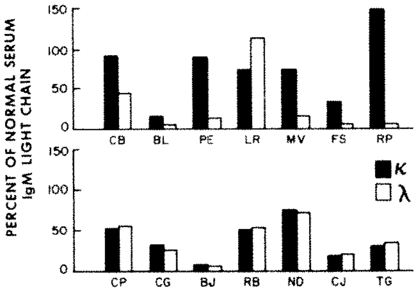

Fig 3. Results of the κ/λ ratio assay. Patients with serum idiotype levels of >15 μg/mL (top) and patients with serum idiotype levels of <15 μg/mL (bottom) are shown, with increasing idiotype levels from left to right. The minimum serum idiotype among the >15 μg/mL group was 100 μg/mL (C.B.).

DISCUSSION

Because a custom-made antibody must be made for each patient, criteria must be established that will facilitate the selection of the most favorable candidates for anti-idiotype therapy. Circulating antigen interferes with therapy using antibodies.[4,6,9,19] This has been a particularly important problem in studies using anti-idiotype antibodies directed against the Ig present on B cell tumors.[4,6,9] Patients with insurmountable levels of idiotype in their serum should be excluded from therapeutic trials. In the past, an anti-idiotype antibody first had to be produced; it could then be used to measure the level of circulating idiotype protein. In this study, we developed in vitro assays that predict which patients have significant levels of serum idiotype without using an anti-idiotype reagent.

We prepared monoclonal anti-idiotype antibodies for all the patients studied in this report except D.H. This enabled us to measure idiotype levels accurately in these patients to establish a correlation with in vitro predictive tests. Several factors may have influenced both the in vitro assay results and the in vivo idiotype levels. The number of cells added to each well of the in vitro assays was based only on viable cells, with no adjustments made for the cellular composition, ie, the ratio of tumor, normal B cells, and normal T cells. Thus, the number of Ig-secreting cells in each well were proportionately reduced in tumors with large numbers of T cells. In addition, the T cells may influence, either positively or negatively, the ability of the malignant B cells to secrete Ig. Finally, the in vivo level of serum idiotype varies directly with tumor bulk.[11] Despite these theoretical problems, the in vitro assays reliably predicted in vivo levels.

The effects of various mitogens, including PHA and phorbol esters, on secretion of immunoglobulin by normal and neoplastic B cells were previously studied in vitro.[20-28] PHA added to cultures of normal T and B cells caused proliferation of both sets of cells, expression of interleukin 2 (IL2) receptor on the T cells, and increased secretion of Ig by B cells. Most of these effects were mediated by soluble B cell growth and differentiation factors secreted by the stimulated T cells. The mitogens produced both increased secretion of Ig and maturation of the malignant cells, as measured by switch of idiotype-positive IgM and IgD to IgG.[21,22] No correlation with the in vivo secretion of the malignant cells has been previously reported. Although a direct effect of the mitogens on the tumor cells produced some enhancement of secretion, their effect on T lymphocytes, also present in the culture, was probably more important.[23] Defects in the in vitro function of the T lymphocytes of patients with B cell malignancies have been identified.[23-28] Some reports showed that allogeneic cells were far better at stimulating secretion of Ig than were autologous T cells.[23,25,26] Indeed, suppression was induced by adding back autologous T cells to a culture containing normal allogeneic T cells and the malignant B cells.[26] Other investigators were unable to identify abnormal T cell suppression.[27,28] We found no correlation between the numbers or types of T cells and spontaneous or induced Ig secretion among the tumors in this study.

The autologous T cells may have suppressed production in the patients whose tumors secreted little or no Ig in vitro or in vivo. Among those that secreted Ig in vitro, however, our data suggested an enhancing effect of T cell stimulation on Ig secretion. Differences were evident among the patients regarding the necessity for PHA and/or PMA to produce maximal secretion of Ig. In patients R.P. and L.R., PMA and PHA had a synergistic effect on idiotype secretion. PMA induces IL 2 receptor expression on T cells.[26] This effect, together with the T cell proliferation induced by PHA, may have led to amplification of synthesis and secretion of B cell growth and differentiation factors. Patient R.P. had the highest level of secretion in vitro (Table 2, >3.0 μg/mL), but only after stimulation with both PMA and PHA. Serum protein electrophoresis (SPEP) showed that this patient had a paraprotein spike of 20 mg/mL, suggesting that T cells were necessary for tumor idiotype secretion in vivo as well as in vitro. Similar observations were made for L.R. and M.V., who also had high levels of serum idiotype. Previously, we reported that the absolute number of T cells in the tumor correlated positively with the patient's response to anti-idiotype therapy.[15] T cells located within the tumor or elsewhere may regulate the in vivo secretion of idiotype as well as the control of growth and differentiation of the malignant B cells.

The assay to measure spontaneous and induced secretion of Ig was designed to correlate with in vivo secretion and thus with serum idiotype. The effects of autologous T cells were not eliminated in this assay, so that we could simulate the in vivo situation. Although this assay measured free light chains and IgG as well as IgM, we believe that quantitation of the total Ig secreted in vitro is important in determining the in vivo secretory capacity of the tumor cells. The assay was successful in this respect. The spontaneous or induced secretion of idiotype was able to identify patients with high serum idiotype levels (Fig 1). In vitro secretion <0.5 μg/mL can be used to select patients with little or no circulating idiotype. Conversely, in vitro secretion of >1.0 μg/mL identifies patients with high levels of serum idiotype who are not good candidates for anti-idiotype therapy.

We also tested an assay that measures the relative imbalance of IgM light chains in the serum to detect circulating IgM tumor idiotype. The advantage of this test is that culture of cells is not required and results may be obtained rapidly. Although the assay successfully identified patients with large amounts of IgM, its lack of sensitivity and inapplicability to IgG-secreting tumors limited its usefulness.

Other workers have isolated tumor idiotype either from serum or from cultures of malignant cells.[26] Although these techniques simplify the process of making anti-idiotype antibodies, it requires patients with high levels of serum idiotype. Thus, these methods have the disadvantage of selecting for the most unfavorable patients.

Our studies have considerable practical significance. The ability to select patients with low levels of serum idiotype will facilitate therapeutic studies using anti-idiotype. Our studies may also be useful in monitoring tumor activity and response to treatment. In addition to their practical importance, our studies have important implications regarding the immunoregulation of B cell tumors.

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CONCISE REPORT

Mother-to-Offspring Transmission of Human T Cell Leukemia Virus Type I in Rabbits

By Yoshiki Uemura, Shigemitsu Kotani, Shizuo Yoshimoto, Masatoshi Fujishita,
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We studied the mode of natural transmission of human T-cell leukemia virus type I (HTLV-I) in rabbits. Four virus-infected rabbits (2 males and 2 females) were individually mated with 4 noninfected rabbits. Two virus-infected females mated with noninfected males gave birth to 7 offspring, and 2 noninfected females mated with infected males delivered 5 offspring. Four of the seven offspring born to the virus-infected mothers seroconverted for HTLV-I when aged 6 to 13 weeks with antibody titers of 1:40 to 1:160. None of the five offspring born to the noninfected mothers became seropositive during the observation period of 6 months, however. Peripheral lymphocytes were cultured with T cell growth factor, and HTLV-I-carrying lymphoid cell lines were established from the four seroconverted rabbits. All four cell lines were of T cells positive for Ia antigens. In addition, none of five newborn rabbits killed immediately after birth to a virus-infected rabbit was infected with HTLV-I. These findings provide an experimental support for the milkborne transmission of HTLV-I from mother to child in humans and indicate that the virus is tropic for T cells in rabbits as well.

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THE HUMAN T-cell leukemia virus type I (HTLV-I) is an exogenous type C retrovirus etiologically associated with adult T cell leukemia (ATL).[1] The virus is transmissible by blood transfusion.[2] Seroepidemiologic surveys suggest natural transmission of HTLV-I from mother to child and between spouses.[3,4] Detection of viral antigens in semen and mothers' milk indicate that the virus was transmitted by sexual contact and by breast feeding.[5,6] Recently, HTLV-I was shown to be transmissible to marmosets by oral inoculation of virus-infected lymphocytes from ATL patients[7] and by feeding of milk from seropositive women.[8] We showed that rabbits could be infected with HTLV-I intravenously,[9] orally,[10] and by blood transfusion.[11] We now report natural transmission of HTLV-I from mother to offspring in this animal model.

MATERIALS AND METHODS

Animals. Non-inbred Japanese white rabbits weighing ~3 kg were used. They were purchased from a commercial breeder and received pelleted diet and tap water. Two females and two males were infected with HTLV-I by transfusion of 20 mL of blood from virus-infected rabbits, as described previously.[11] All four rabbits seroconverted for HTLV-I after 2 to 4 weeks, and their antibody titers ranged from 1:80 to 1:640 in an immunofluorescence assay. The four virus-infected rabbits were housed individually and were mated with four noninfected rabbits.

Detection of antibodies to HTLV-I. Rabbits born to the virus-infected parents were followed for the development of antibodies to HTLV-I. Blood samples were taken first when the rabbits were aged 4 weeks and then serially at intervals of 1 to 2 weeks for 6 months. Sera were titrated for HTLV-I antibodies by indirect immunofluorescence[12] using the MT-2 cell line.[13] In brief, acetone-fixed MT-2 cells were first reacted with appropriately diluted rabbit sera and then with fluorescein-conjugated (FITC-conjugated) swine anti-rabbit immunoglobulins (DAKO, Copenhagen) for 30 minutes at 37°C. Anti-HTLV-I positive and negative rabbit reference sera were included as parallel controls.

Cell culture. Lymphocytes were separated from 10 mL of blood by Ficoll-Hypaque gradient centrifugation. The cells were cultured at 1×10^6/mL in 35-mm Petri dishes with RPMI 1640 medium supplemented with 20% fetal calf serum (FCS), 10% crude T cell growth factor (TCGF) (Japan Immunoresearch Laboratories, Takasaki), and antibiotics. The cultures were incubated at 37°C in a humidified 7.5% CO_2 atmosphere and fed twice a week.

Analysis of surface and antigen markers. Cells were examined for sheep erythrocyte (E) receptors, as described previously.[14] Surface immunoglobulins (SIg) were tested by reacting cells with FITC-conjugated swine anti-rabbit immunoglobulins. They were also tested by membrane immunofluorescence for reactivities with monoclonal antibodies, including Leu-1 (human T cells), OKIa1 (human Ia antigens), L11/135 (rabbit T cells),[15] and 2C4 (rabbit Ia antigens).[16] HTLV-I antigens were detected by indirect immunofluorescence with reference ATL patient's serum and anti-p19[17] and anti-p24[18] monoclonal antibodies.

RESULTS

Seroconversion in offspring of HTLV-I–infected rabbits. Four pairs of male and female rabbits each delivered one litter of 1 to 6 newborns. Two virus-infected females mated with noninfected males gave birth to 7 offspring, and 2 noninfected females mated with virus-infected males gave birth to 5 offspring (Fig 1). Four of the 7 rabbits born to the virus-infected mothers seroconverted for HTLV-I at 6 to 13 weeks after birth. Their antibody titers, initially low at 1:10, rose to the highest levels of 1:40 to 1:160 over the following 2 to 8 weeks and remained at those levels thereafter (Fig 2). In contrast, none of the 5 rabbits born to the noninfected

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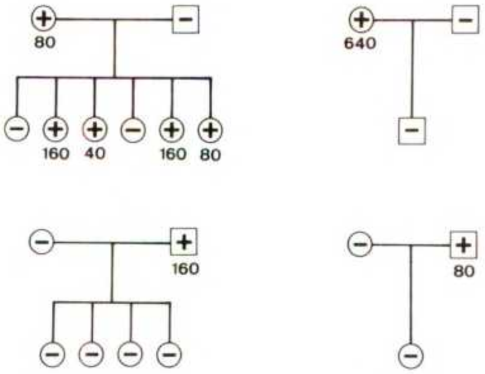

Fig 1. Parent-to-offspring relationship with respect to seroconversion (+) in rabbits. Males (□); Females (○). Numbers below symbols denote final antibody titers to HTLV-I. Seroconverted offspring (left to right) yielded HTLV-I–carrying lymphoid cell lines Ra-7 to Ra-10, respectively.

mothers became seropositive during the observation period of 6 months.

Isolation of HTLV-I from seroconverted offspring. When the rabbits were aged 3 to 4.5 months, blood samples were taken from the seven offspring born to the virus-infected mothers; their lymphocytes were cultured in the presence of TCGF. Active cell growth occurred after 2 to 4 weeks and a TCGF-dependent lymphoid cell line was established from each of the four seroconverted offspring, whereas the cells from the three seronegative offspring did not grow for >2 months. These four cell lines, designated Ra-7 to Ra-10, grew in suspension with clumps of cells and consisted of primitive lymphoid cells (Fig 3). Two of the four cell lines became TCGF-independent after 2 months. The four cell lines had a normal female rabbit karyotype corresponding to the sex of the donors. All four cell lines were reactive with L11/135 and 2C4 but were completely negative for E receptors, SIg, Leu-1, and OKIa1. HTLV-I antigens were fully expressed in all the cell lines as detected with reference ATL patient's serum, anti-p19, and anti-p24. Type C virus particles were demonstrated in these cell lines by electron microscopy (Fig 4). These characteristics are shown in Table 1.

Examination of newborn rabbits for HTLV-I infection. A litter of five newborn rabbits was killed immediately after birth to a virus-infected rabbit, and their splenic lymphocytes were cultured in the presence of TCGF. All five cultures were negative for HTLV-I antigens when examined by indirect immunofluorescence after 3 to 4 weeks and eventually failed to be established as lymphoid cell lines.

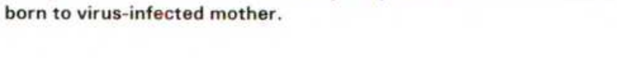

Fig 2. Representative antibody response curve in a rabbit born to virus-infected mother.

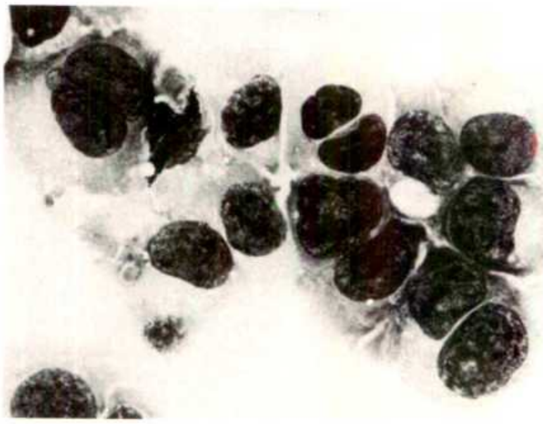

Fig 3. Light-microscopic appearance of Ra-9 cells. Cells vary in size with occasional giant cells. Nucleoli are seen in some cells. (May-Grunwald-Giemsa, original magnification ×1,200; current magnification ×840.)

DISCUSSION

The concept of mother-to-child transmission of HTLV-I has been previously supported on seroepidemiologic grounds[3,4] by the detection of viral antigens in milk from seropositive women,[5,6] and by experimental oral transmission of HTLV-I into marmosets.[7,8] A rabbit model of HTLV-I infection provided a unique opportunity to investigate the mode of transmission of this virus.[9-11] In the present experiment, virus-infected female rabbits were mated with noninfected male rabbits and vice versa, and their offspring were followed for evidence of virus infection. Virus transmission occurred in offspring born to virus-infected mothers but not in offspring born to noninfected mothers. These findings give experimental support for the natural transmission of HTLV-I from mother to child in humans.

Two routes of virus transmission, transplacental or milk-borne, may be considered. To clarify this point, we attempted

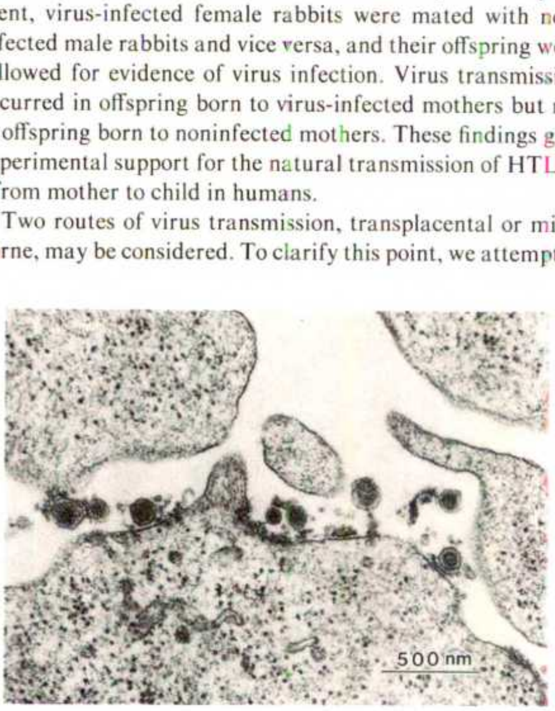

Fig 4. Electron micrograph of Ra-8 cells showing type C virus particles in the extracellular space. (Uranyl acetate and lead citrate, original magnification ×36,000; current magnification × 25,200.)

Table 1. Characteristics of HTLV-I-Carrying Rabbit Lymphoid Cell Lines

Markers	Ra-7	Ra-8	Ra-9	Ra-10
E receptors	0	0	0	0
SIg	0	0	0	0
Leu-1	0	0	0	0
OKIa1	0	0	0	0
L11/135	73	80	82	89
2C4	93	90	100	87
HTLV-I antigens	75	80	90	90
HTLV-I particles	+	+	+	+
Karyotype	44, XX	44, XX	44, XX	44, XX

Numbers are percentage of positive cells.

a foster-nursing experiment by exchanging newborn rabbits between virus-infected and noninfected females. Two litters from virus-infected rabbits and one litter from a noninfected rabbit were all cannibalized after foster-nursing, however. We then examined whether newborn rabbits born to a virus-infected rabbit were already infected at birth with HTLV-I, but the virus could not be detected or isolated from cultures of their splenic lymphocytes. This is consistent with the absence of HTLV-I antigens in cord lymphocytes from neonates born to seropositive women.[4,6] Thus, milkborne rather than transplacental transmission of HTLV-I appears more likely. Previously, we demonstrated that only one of four adult rabbits could be infected with HTLV-I by twice-weekly oral inoculation of a large number of virus-infected cells.[10] Those results, compared with the present data, indicate that suckling rabbits may be more susceptible to oral transmission of HTLV-I.

HTLV-I is known to preferentially transform T cells from monkeys,[19] cats,[20] and rats,[21] as well as from humans.[13,22] Therefore, we wished to determine the lineage of the rabbit lymphoid cell lines transformed by this virus. Recently, monoclonal antibodies to rabbit T cells (L11/135)[15] and rabbit Ia antigens (2C4)[16] became available, and all four cell lines (Ra-7 to Ra-10) were reactive with both monoclonals in the absence of B cell markers. Thus, T cell tropism of HTLV-I appears to be a distinct feature common to a wide range of animal species.

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CONCISE REPORT

Expression of the Macrophage-Specific Colony-Stimulating Factor in Human Monocytes Treated With Granulocyte-Macrophage Colony-Stimulating Factor

By Junko Horiguchi, M. Kim Warren, and Donald Kufe

The macrophage-specific colony-stimulating factor (CSF-1, M-CSF) regulates the survival, growth and differentiation of monocytes. We have recently demonstrated that phorbol ester induces expression of CSF-1 in human monocytes. These findings suggested that activated monocytes are capable of producing their own lineage-specific CSF. The present studies demonstrate that the granulocyte-macrophage colony-stimulating factor (GM-CSF) also induces CSF-1 transcripts in monocytes. Furthermore, we demonstrate that the detection of CSF-1 RNA in GM-CSF–treated monocytes is associated with synthesis of the CSF-1 gene product. The results thus suggest that GM-CSF may indirectly control specific monocyte functions through the regulation of CSF-1 production. These findings indicate another level of interaction between T cells and monocytes.

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COLONY-STIMULATING FACTORS control the in vitro proliferation and differentiation of hematopoietic progenitor cells.[1,2] These factors include granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF), macrophage-CSF (CSF-1, M-CSF) and multi-CSF (interleukin 3).[1] A cDNA for the T lymphocyte–derived GM-CSF has been cloned and recombinant GM-CSF protein purified to homogeneity.[2] This purified human GM-CSF has recently been shown to induce tumoricidal activity in human peripheral blood monocytes.[3] The effects of GM-CSF on specific gene expression in monocytes, particularly that of other CSFs, however, remains unclear.

CSF-1 is required for the survival, growth, and differentiation of monocytes.[4] The effects of CSF-1 are mediated by binding to the CSF-1 receptor, a 165-kd glycoprotein encoded by the c-*fms* protooncogene.[5] We[6] and others[7] previously demonstrated that the c-*fms* gene is expressed in cells differentiated along the monocytic lineage. More recent studies have demonstrated that CSF-1 is also expressed during phorbol ester–induced monocytic differentiation.[8,9] These findings suggested that monocytes may have the capacity to autostimulate certain functions through the production of CSF-1. Our previous studies, however, did not examine whether specific cytokines are capable of regulating CSF-1 expression in monocytes.

MATERIALS AND METHODS

Cell culture. Human peripheral blood monocytes were purified by Ficoll-Hypaque separation, adherence for one hour and then removal of the nonadherent cells.[10] The adherent cell population was collected with a plastic policeman, readhered overnight, and washed again with four changes of medium. The second adherent cell population consisted of over 98% monocytes by morphologic examination. Monocyte purification and culturing were performed in RPMI 1640 medium with 10% pooled human AB serum.[10] The purified monocytes were treated with 20, 100, 500, and 2,500 CFU-C/mL GM-CSF (specific activity: 5×10^4 CFU-C/μg).[2,3] The recombinant human GM-CSF was produced in yeast and was free of detectable endotoxin by the limulus assay.

DNA probes. The 1.6-kb fragment of a human CSF-1 cDNA was purified from the pcCSF-12 plasmid.[11] The 1.0-kb *Pst*I fragment of the v-*fms* gene was purified from the pSM3 plasmid.[6]

Hybridizations. The procedures used for RNA extraction, transfer, hybridization, and washing were as reported previously.[6]

CSF-1 determinations. The concentration of CSF-1 in tissue culture supernatants was determined by the CSF-1–specific radioimmune assay.[11]

RESULTS

We have previously shown that TPA treatment of human peripheral blood monocytes induces CSF-1 expression.[8] A maximum increase in CSF-1 RNA was achieved six hours after TPA induction and these transcripts declined thereafter. Treatment of resting human monocytes with GM-CSF similarly induced CSF-1 expression. The CSF-1 cDNA probe hybridized with a 4.6-kb transcript detectable at three hours of exposure to 500 CFU-C/mL GM-CSF (Fig 1). Similar findings have been obtained using poly A–selected monocyte RNA (data not shown). Furthermore, the level of CSF-1 RNA induced by GM-CSF remained detectable through 48 hours. In contrast, CSF-1 transcripts were undetectable following the same analysis of monocytes incubated in the absence of GM-CSF. The findings in GM-CSF–treated monocytes were also associated with a partial down-regulation of c-*fms* expression (Fig 1). Similar effects on CSF-1 and c-*fms* expression were obtained following treatment of monocytes with 20, 100, and 2,500 CFU-C/mL GM-CSF (data not shown).

We have also monitored production of the CSF-1 protein in GM-CSF–treated monocytes. CSF-1 levels in monocyte supernatants increased nearly tenfold at three hours of GM-CSF exposure (Fig 2). Furthermore, the CSF-1 levels remained elevated at 48 hours of treatment. In contrast, monocytes incubated in the absence of GM-CSF had no detectable supernatant CSF-1 at similar intervals and at 72 hours. Thus, CSF-1 expression at the RNA level corre-

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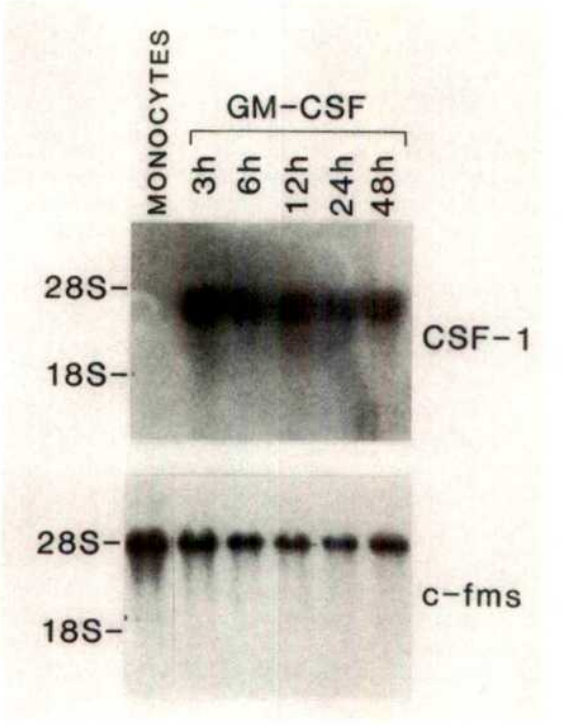

Fig 1. Levels of CSF-1 and c-*fms* RNA during treatment of human monocytes wity GM-CSF. Peripheral blood monocytes wre purified as described in Materials and Methods and treated with 500 CFU-C/mL GM-CSF. Total cellualr RNA was purified by the guanidine thiocyanate–cesium chloride method, analyzed by electrophoresis of 12 μg RNA through 1% agarose-formaldehyde gels followed by Northern blot transfer to nitrocellulose. The RNA filters were hybridized to the ^{32}P-labeled nick-translated CSF-1 and v-*fms* probes.

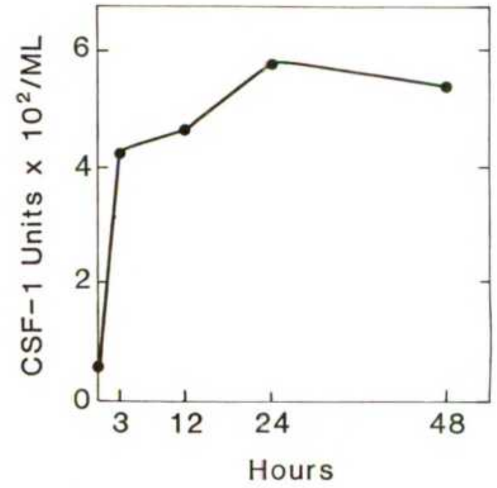

Fig 2. CSF-1 levels in tissue culture supernatants of GM-CSF treated monocytes. Monocytes were treated with GM-CSF as described in the legend to Fig 1. The supernatants were assayed for CSF-1 using the CSF-1-specific radioimmune assay.[11] The values represent the CSF-1 concentration for 1×10^8 monocytes/mL. The value shown for the zero time point represents the lower limit of this assay.

sponded with detection of the CSF-1 protein in culture supernatants.

DISCUSSION

The present results demonstrate that GM-CSF induces CSF-1 expression in human monocytes. This induction occurred during the continued expression of c-*fms* transcripts. Since c-*fms* encodes for the CSF-1 receptor, the findings would suggest that GM-CSF treated monocytes may regulate their own growth and differentiation through CSF-1 production. Thus, the in vitro effects of GM-CSF on macrophage colony formation[1] may be mediated in part by induction of CSF-1 expression. Furthermore, recent studies have demonstrated that CSF-1 also stimulates human monocyte production of interferon, tumor necrosis factor, and myeloid colony-stimulating activity.[12] Taken together, these findings would suggest that GM-CSF, and therefore T lymphocytes, may indirectly control specific functions of monocytes through the regulation of CSF-1 expression.

Other cytokines appear capable of inducing CSF-1 in monocytes. Previous studies have demonstrated that T cell–derived gamma-interferon induces release of monocyte colony-stimulating activity from purified human monocytes.[9,13] Thus, gamma-interferon and GM-CSF may both contribute to the interaction between T cells and monocytes by inducing CSF-1 expression. Furthermore, our studies have not addressed the possibility that GM-CSF first induces expression of a monokine, which in turn regulates CSF-1 production. In this regard, recent studies have demonstrated that both tumor necrosis factor and interleukin 1 are capable of stimulating monocyte cytotoxicity.[14] The effects of these monokines on CSF-1 expression in human monocytes will require further study.

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CONCISE REPORT

Bone Marrow Transplantation in Multiple Myeloma: Report From the European Cooperative Group for Bone Marrow Transplantation

By G. Gahrton, S. Tura, M. Flesch, A. Gratwohl, P. Gravett, G. Lucarelli, M. Michallet, J. Reiffers, O. Ringdén, M.T. van Lint, J.P. Vernant, and F.E. Zwaan

Of 14 patients who received an allogeneic bone marrow graft from HLA-compatible sibling donors, 10 have survived for 6 to 34 months posttransplantation (median, 12 months). Four patients have died, two of relapse at extramedullary sites, one of severe acute GVHD, and one from GI bleeding and pericardial effusion. One patient is alive in relapse and four patients have signs of minimal persistent disease. Five patients are well without signs of active disease. Minor improvement in osteolytic lesions on X-ray were seen in three patients, but the X-ray bone structure was mainly unchanged in most patients. Bone marrow transplantation appears promising for treatment of certain patients with multiple myeloma.

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ALLOGENEIC BONE MARROW transplantation (BMT) has proved to be an efficient method for treatment of patients with a variety of hematological malignancies, in particular acute leukemia and chronic myelocytic leukemia.[1] BMT has also been performed in a few patients with multiple myeloma,[2-6] three of whom have survived more than two years posttransplantation, although they were transplanted in an advanced stage of the disease. Within the European Cooperative Group for Bone Marrow Transplantation (EBMT), 14 myeloma patients have been transplanted. This report describes the outcome for these 14 patients, some of whom have been presented previously in preliminary reports.[5-6]

MATERIALS AND METHODS

Fourteen patients (8 women, 6 men, median age 35 years) (Table 1) were reported to the EBMT registry, having been transplanted for multiple myeloma. All patients had been treated with cytotoxic drugs prior to BMT. Five were in stage III, two in stage II, and seven in stage I prior to the start of consolidation treatment. Five were considered refractory to treatment (patients 4, 6, 7, 8, and 10), while 9 were not refractory to first- or second-line treatment. Five patients had received only melphalan and prednisolone before transplant; two of them were refractory to this treatment, and two were responding. Nine patients had received a combination of various cytotoxic drugs. The time from diagnosis to BMT varied considerably (median 14 months).

All patients were conditioned before BMT with both cyclophosphamide and total body irradiation (modified Seattle protocol).[7] Five patients received additional cytotoxic drugs, ie, CCNU (lomustine), BCNU (carmustine), vincristine, prednisolone, and melphalan in variable regimens. Eight patients received total body irradiation in a single dose and six in fractions over three to five days. The total dose varied between 9.5 and 12 Gy, according to whether it was given as a single dose or in fractions.

All patients were advised of BMT procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent.

RESULTS

Of the 14 patients, 10 are alive 6 to 34 months (median 12 months) post-BMT (Table 1). Four patients died ½ to 4½ months posttransplant. One patient with an IgG-lambda plasma-cell leukemia died with a meningeal relapse 4½ months post-BMT, and one patient with an IgG-kappa myeloma died with a cerebral relapse. In these two patients there were no signs of generalized disease. One patient died from GI bleeding and pericardial effusion, and the fourth patient died of severe GVHD.

Among the surviving patients five were well and without signs of disease. Two of them were considered refractory to the chemotherapy before BMT. They had no abnormal serum immunoglobulins, light chains in the urine, or abnormal plasma cells in the marrow at reporting. Four patients had persistent minimal signs of disease (patients 3, 4, 10, and 14) (Table 1) and one patient relapsed (patient 5).

The pretransplant bone structure on X-ray was only moderately changed posttransplantation. Of the five patients without signs of active disease in serum, urine or bone marrow post-BMT, three had advanced lytic lesions before transplant. After BMT no clear change was observed in one of these patients, while two were rated as having only minor lytic lesions. In the other two patients no change in the bone structure was observed posttransplant (normal bone structure and minor bone lesions, respectively). Of the four patients with minimal disease, two were rated as having

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Table 1. Bone Marrow Transplantation in Multiple Myeloma

						Conditioning				GVHD		
							TBI					
Patient	Sex/Age	Type of Myeloma	Treatment Before BMT	Time Diagnosis—BMT mo	Stage at BMT	Chemo	Total Dose	No. of Days	Survival mo post-BMT	Acute Grade	Chronic	Comments
1	F /29	Plasma cell leukemia IgDλ	M, C+V+P+M+D	7	2	C 120 mg/kg CCNU 340 mg	12.0	3	4½	II	mild	Dead, meningeal relapse
2	F /45	IgGκ	M+P+D+A	18	1	C 120 mg/kg M 140 mg/m^2	12.0	3	3½	0	0	Dead, cerebral relapse
3	F /35	IgAλ	C+P+CCNU+D	7	1	C 120 mg/kg	10.0	1	12+	0	0	Alive, well, minimal disease; persistent S-Ig <0.5 g/L; urinary light chains 0.2 g/L
4	M/35	IgAκ	V+D+C+P	6	2	C 120 mg/kg BCNU 3.5 mg/kg V 2 mg,P60 mg/kg	12.0	3	13½	0	0	Alive, well minimal disease; persistent S-Ig 5g/L
5	F /39	IgAλ	M+C+P	13	1	C 120 mg/kg M 140 mg/m^2	11.0	5	11+	I	0	Alive, relapse
6*	M/32	BJκ	C+V+P+M	25	3	C 120 mg/kg	10.0	1	9+	I	0	Alive, well, no signs of active disease
7*	F /38	IgGλ	M+P	18	3	C 120 mg/kg	10.0	1	½	0	0	Dead, GI bleeding and pericardial effusion
8*	F /46	IgAκ	M+P	5½	3	C 120 mg/kg	10.0	1	34+	I	0	Alive, well, no signs of active disease
9	M/29	BJκ	M+P	8	3	C 120 mg/kg	10.0	3	12+	I	0	Alive, well, no signs of active disease
10	M/44	IgAλ	M+P	8	1	C 120 mg/kg M 4 mg/kg	10.0	1	8+	I	0	Alive, well, minimal disease 10% plasma cells in the bone marrow
11*	F /35	IgGκ	M+P	24	1	C 120 mg/kg	10.5	1	28+	I	mild	Alive, well, no signs of active disease
12*	M/44	IgAκ	M+P M+D+BCNU+C+V	16	3	C 120 mg/kg M 2 mg/kg BCNU 5.5 mg/kg	9.5	1	12+	I	mild	Alive, well, no signs of active disease
13*	M/34	IgAκ	M+P M+P+BCNU+C+V	58	1	C 120 mg/kg M 2 mg/kg BCNU 5.5 mg/kg	10.0	1	2	IV	0	Dead, severe acute GVHD
14	F /38	IgGκ	V+C+M+P V+D+P	14	1	C 120 mg/kg	12.0	3	6+	I	mild	Alive and well, minimal disease; persistent S-Ig 6 g/L

Abbreviations: M, melphalan; C, cyclophosphamide; V, vincristine; P, predniso(lo)ne (or dexamethasone); A, cytosine arabinoside (ARA-C); D, doxorubicin; CCNU, lomustine; BCNU, carmustine.

*Earlier reported after shorter follow-up time (5,6) minor bone lesions before BMT. In one of these patients there was no change; the other had normal bone structure at reporting. Two patients had normal bone structure before transplant. In one there is still no change, while one now has minor bone lesions.

Only one patient had severe (grade IV) acute GVHD (patient 13). No patient had severe chronic GVHD, and four had mild chronic GVHD.

DISCUSSION

This report shows that bone marrow transplantation can be successful in patients with multiple myeloma. Five of 14 patients are well without signs of active disease 9 to 34 months posttransplantation. Four other patients are well with only minimal changes in either serum Ig, urinary light chains, or bone marrow plasma cells, indicating persistent disease. Although the observation time is short (median 12 months for surviving patients), only three patients have relapsed. The nature of minimal persistent disease is uncertain, and these patients may well relapse later. In fact, patient 5 had signs of persistent disease for some months before relapse at 11 months post-BMT. On the other hand, the four patients with persistent minimal disease at reporting have now survived for 6, 8, 12, and 13½ months without progressing. It is possible that these minimal changes may disappear, perhaps due to a graft-versus-myeloma effect as previously described for acute leukemia.[8] The significance of persistent bone lesions on X-ray is difficult to judge. Three of five patients who were well without signs of active disease at this report had advanced osteolytic lesions at transplant. Although one still has advanced osteolytic lesions and the other two minor ones, none has signs of active disease 9 to 12 months post-BMT. One patient who had minor osteolytic lesions unchanged post-transplant has now survived for 34 months. Thus, persistent osteolytic lesions may not indicate a risk for relapse.

Side effects of BMT in this material were in general not different from those described previously.[1] It is of interest to note, however, that only one patient had severe acute GVHD (T cell–depleted) and none had severe chronic GVHD. Thus, despite the relatively advanced age of the patients, GVHD was a minor problem.

The optimal time for transplantation of patients with multiple myeloma cannot be judged from the present report. However, it seems reasonable to transplant patients with an HLA-compatible sibling donor if the prognosis is poor with current chemotherapy. Recently some prognostic factors have been delineated,[9] ie, patients with a rapid response to treatment, with IgD myeloma, and with a high thymidine-incorporation labeling index appear to have a relatively poor prognosis. These patients could perhaps be transplanted at an early stage of the disease. Also, since two out of five patients considered refractory to chemotherapy were without signs of active disease 9 and 34 months post-BMT, patients that are resistant to first-line treatment may well be successfully transplanted before second-line treatment is instituted.

APPENDIX

This study was based on reports from the following centers within the European Cooperative Group for Bone Marrow Transplantation (EBMT): Huddinge Hospital, Huddinge, Sweden (G. Gahrton, O. Ringdén, B. Lönnqvist, and P. Ljungman), University Hospital, Bologna, Italy (S. Tura), Hospital Jean-Minjoz, Besancon, France (M. Flesch), Cromwell Hospital, London, UK (P.J. Gravett and D. Guéret-Wardle), Pesaro Hospital, Pesaro, Italy (G. Lucarelli), University Hospital, Grenoble, France (M. Michallet, B. Corront, and D. Hollard) University Hospital, Pessac, France (J. Reiffers, G. Marit, and B. David) Hospital S Martin, Genova, Italy (M.T. van Lint), and University Hospital, Creteil, France (J.P. Vernant).

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CONCISE REPORT

Monocyte Adhesion to Subendothelial Components

By John W. Tobias, Murray M. Bern, Peter A. Netland, and Bruce R. Zetter

Human monocytes have been shown to penetrate the endothelial layer of large blood vessels and to adhere to the subendothelial basement membrane. To determine the active components of this process, we have studied the ability of monocytes to adhere to isolated components of the subendothelial matrix. Using a quantitative dot-blot adhesion assay, we find that monocytes adhere preferentially to immobilized laminin and elastin. The monocytes adhere less well to fibronectin and bind poorly or not at all to collagen types I and IV, or to heparan sulfate. Monocyte binding to elastin requires an intact, crosslinked molecule as no binding was observed to soluble, acid-alcohol elastin extracts, to pepsin or elastase digests of elastin, to tropoelastin monomer, or to desmosine/isodesmosine crosslinks. Similar binding profiles to elastin, laminin, and fibronectin were seen with the established human leukocyte cell line U937. The promyelocytic cell line HL60 adhered equally well to laminin but showed slightly reduced adhesion to elastin when compared with the fresh monocytes or U937 cells. Freshly isolated human erythrocytes did not demonstrate significant adhesion to fibronectin, laminin, or elastin.

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IT HAS RECENTLY BEEN demonstrated that blood-borne mononuclear cells can penetrate the vascular endothelium in large blood vessels and adhere to the subendothelial basal lamina.[1-3] It has been suggested that these monocytes are the precursors of the lipid-laden foam cells associated with atherosclerotic lesions.[4,5] Monocyte-derived growth factors may also mediate the smooth muscle cell migration and proliferation that is an early step in the pathogenesis of atherosclerosis.[6,7]

The molecules that mediate monocyte adhesion to the subendothelial extracellular matrix have not yet been determined. It is possible that there are multiple determinants in the extracellular matrix (ECM) to which monocytes can adhere. To explore this question, we have tested several known components of the vascular ECM for their ability to bind monocytes. Our results demonstrate that monocytes and related leukocyte cell lines adhere preferentially to laminin and elastin. The cells adhere less well to fibronectin and do not bind appreciably to other matrix components.

MATERIALS AND METHODS

Isolation of human peripheral blood monocytes. Whole blood from a fasting donor was collected in Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing 0.1 mL of 15% ethylenediaminetetraacetic acid. One mL of 6.0% Dextran T500 (Pharmacia Fine Chemicals, Piscataway, NJ) in Ca++- and Mg++-free phosphate buffered saline (PBS, GIBCO, Grand Island, NY) was added to every 9 mL of whole blood. After the erythrocytes had been allowed to settle for 35 to 45 minutes at room temperature, the leukocyte-rich plasma was removed and layered in 6-mL aliquots over 3 mL of Nycodenz (Accurate Chemical & Scientific, Westbury, NY) adjusted with 1.02% NaCl to a final density of 1.067 g/mL and an osmolarity of 330 mosmol/L according to the method of Bøyum.[8] After centrifugation at 2,000 × g for 15 minutes, the interface and the upper 1 mL of the bottom layer were collected. Approximately 2×10^5 mononuclear white blood cells were obtained per mL of whole blood. The collected cells were shown to be >92% monocytes by the criteria of morphology and nonspecific esterase activity.[9] In some experiments, erythrocytes were collected from the same blood samples.

Other leukocyte cell lines. U937 cells, a human lymphoma-derived cell line with monocytelike properties[10] and HL60, a human promyelocytic cell line[11-14] were generously provided by Dr Michael Bevilacqua, Brigham and Women's Hospital, Boston, MA. Both cell lines were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO).

Cellular radiolabeling. Cells were suspended at a concentration of 2×10^6 cells/mL in PBS containing 0.5% bovine serum albumin (Boehringer Mannheim, Indianapolis, IN) and 200 μCi/mL sodium ^{51}chromate (Dupont–New England Nuclear, Boston, MA). The suspension was incubated for one hour at 37°C with occasional stirring. The suspension was then brought to a volume of 10 mL in Ca++- and Mg++-free PBS and centrifuged at 800 × g for 10 minutes. The pellet was washed twice in 10 mL PBS and brought to a final concentration of 2×10^5 cells/mL in HEPES-buffered Medium 199 (GIBCO) and an aliquot was counted in a Beckman (Irvine, CA) 5500 gamma counter. The labeled cells had an average activity of 0.09 to 0.16 dpm/cell.

Adhesion assay. The cell adhesion assay employed in these studies was performed as previously described.[15] Briefly, adhesion molecules were adsorbed under vacuum to 4-mm dots on Zeta-Probe nylon membranes (Bio-Rad, Richmond, CA) in a Schleicher and Schuell (Keene, NH) "Minifold" apparatus. The nylon membrane was removed from the Minifold and cut into squares containing four dots per square. These squares were then placed into individual wells of a Costar 12-well culture dish (Bellco Glassware, Vineland, NJ) and incubated at 37°C for 18 hours in PBS containing 0.5% bovine hemoglobin (Sigma, St Louis) to block sites on the membrane not occupied by the test molecule. The membranes were then washed twice with hemoglobin-free PBS and once with Medium H-199. Each well then received 2 mL of serum-free medium H-199 containing 2×10^5 cells/mL. They were then incubated for 25 minutes at 37°C on a rotary shaker rotating at 50 rpm (American Rotator V.

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American Scientific Products, Miami, FL). After the incubation, the membranes were washed twice gently in 4°C PBS to remove unbound monocytes and then fixed in 1.0% glutaraldehyde (Eastman, Rochester, NY) for 30 minutes at room temperature. The membranes were finally rinsed twice in PBS and dried overnight in a vacuum desiccator. Binding of the monocytes was visualized by exposing the membranes to Kodak XAR-5 X-ray film for two to three days at −20°C (Fig 1B). Binding was quantified by punching out the individual dots and counting the associated radioactivity.

Reagents. Mouse lung elastin was purified in our laboratory according to the method of Barnard et al.[16] Bovine aortic elastin, purified according to the Starcher method[17] was obtained from Elastin Products Company (EPC, Pacific, MO). Amino acid analysis of the elastin preparations, performed by Dr David Ander, Harvard Microchemistry Facility, Cambridge, MA,[15] yielded profiles consistent with that of purified elastin according to the criteria of Soskel and Sandberg.[18] Alpha, kappa and kappa-2 elastin hydrolysates as well as desmosine/isodesmosine were from EPC. Tropoelastin monomer was purified from neonatal rat aortic arch smooth muscle cells according to the method of Sandberg as modified by Chipman et al.[19] Collagen types I and IV were generously provided by Dr Helene Sage, University of Washington, Seattle, WA. Samples of laminin and fibronectin were gifts of Dr Donald Ingber, Children's Hospital, Boston, MA. Chondroitin sulfate was from Sigma. Heparan sulfate was from Diosynth, Chicago, IL.

RESULTS

Isolated components of the subendothelial extracellular matrix were adsorbed to 4-mm dots of nylon-based membrane and tested for their ability to mediate monocyte attachment. When blocked with BSA or hemoglobin, the membrane itself had no significant monocyte binding activity. Monocytes did, however, adhere to elastin or laminin that had been adsorbed onto the nylon membranes (Fig 1). The binding to each of the two molecules was dose-dependent and did not saturate at doses below 100 μg/dot. Fewer monocytes bound to membranes containing 75 μg/dot fibronectin than to equivalent doses of laminin or elastin. Other ECM components were tested for their ability to mediate monocyte adhesion. No adhesion activity was seen with heparan sulfate, chondroitin sulfate, or with collagen types I or IV (Fig 2). Control experiments with ^{3}H-elastin, laminin, and fibronectin that had been labeled by reductive methylation with sodium borohydride[20] revealed no significant difference in the binding of any of these factors to the nylon membrane (not shown). In each case, greater than 90% of the molecules bound at all doses below 100 μg/dot. Above this concentration, the membranes became saturated and binding was variable.

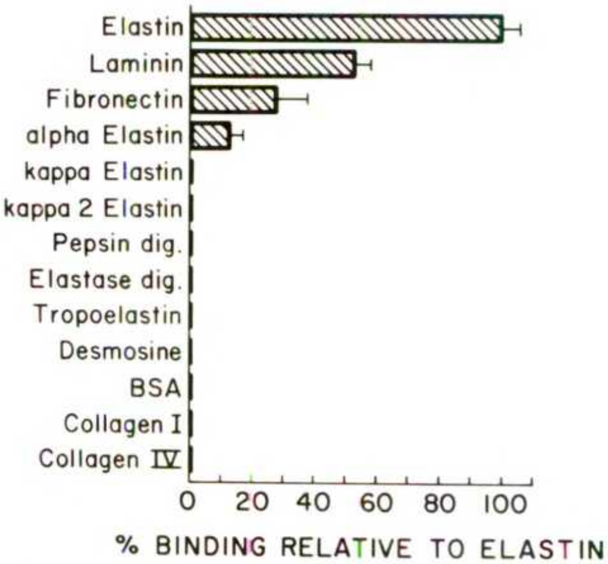

Fig 2. Comparative binding of human peripheral monocytes to extracellular matrix components. Seventy-five μg of each molecule was adsorbed to 4-mm dots on nylon membranes and the membranes were then incubated with radiolabeled monocytes. Data are expressed as the percent binding relative to elastin (mean ± SD, N = 4).

In situ, elastin exists as a hydrophobic, highly crosslinked, insoluble molecule.[21,22] To determine whether the structure of mature crosslinked elastin was required for its adhesive properties, we tested several preparations of solubilized elastin for their adhesive potential. No significant activity was observed with hydrolysates of elastin that had been prepared by incubating bovine aortic elastin with pepsin or with pancreatic elastase or by treatment with acid to make alpha elastin[23] or with alkali to make kappa or kappa-2 elastin.[24] Monocytes also failed to adhere to tropoelastin monomer isolated from newborn rat aortic arches. Finally, no adhesion was observed to desmosine/isodesmosine, a synthetic preparation of the crosslinking region specific to elastin.[25] Together, these results suggest that the adhesive activity of the mature elastin molecule is dependent on an intact cross-linked molecule and cannot be duplicated by tropoelastin or by hydrolytic fragments of the parent molecule.

Two established human cell lines were also tested for their ability to bind to extracellular matrix components: U937 cells originally derived from a diffuse histiocytic lymphoma[10]

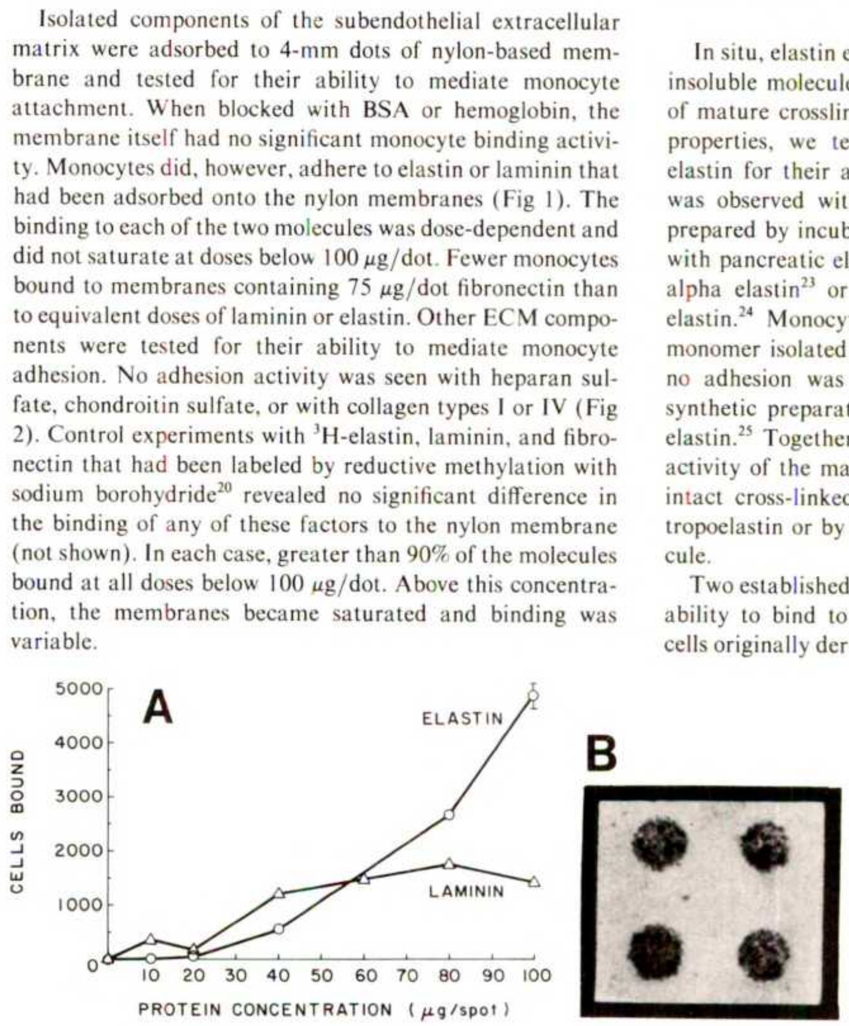

Fig 1. (A) Dose response of monocyte binding to laminin and elastin. Elastin and laminin were adsorbed at the indicated concentrations to 4-mm dots on nylon membranes. After incubation with radiolabeled cells, the number of adherent cells was measured as a function of the radioactivity associated with each dot. Data points represent the mean of four separate determinations. (B) Autoradiography of dots with adherent monocytes.

and the promyelocyte cell line HL60.[11-14] The monocytelike U937 cells have a binding affinity nearly identical to that of the freshly isolated monocytes with maximal binding to elastin followed by laminin and fibronectin (Fig 3). The HL60 cells also bound to these three molecules showing slightly greater adhesiveness to fibronectin and slightly reduced adhesiveness to elastin when compared with the freshly isolated monocytes or the U937 cells. Human erythrocytes, in contrast, failed to show significant binding to elastin, laminin, or fibronectin.

DISCUSSION

Components of the vascular extracellular matrix have been shown to be adhesion molecules for a variety of cell types.[26,27] Because of recent interest in the adhesion of monocytes to the subendothelial vascular basement membrane,[2] we attempted to elucidate the nature of the cell adhesion molecules in the vessel wall that mediate monocyte adhesion. Our results demonstrate that freshly isolated human peripheral blood monocytes adhere strongly to laminin and elastin. Fibronectin was a less effective monocyte adhesin, and neither heparan sulfate, nor collagen types I and IV had appreciable activity. An established human cell line with monocytelike properties (U937) and a human promyelocytic cell line (HL60) had similar adhesive specificities, whereas human erythrocytes failed to bind markedly to either elastin, laminin, or fibronectin.

It has been shown previously that mononuclear cells interact with elastin. Monocytes release a proteolytic enzyme that has potent elastolytic activity.[28,29] Furthermore, the elastin fragments produced by proteolytic cleavage of mature elastin have chemotactic activity for monocytes.[30] It has been postulated that these activities allow monocytes to invade and migrate across the elastic components of blood vessels in the process of inflammatory extravasation. To these interactions we now add a third, the adhesion of monocytes to intact elastin.

Monocytes bind to elastin in a dose-dependent fashion. No adhesive activity is associated with soluble noncrosslinked tropoelastin monomer or with fragments of elastin produced by protease digestion or by extraction in acid or alkali even though such fragments have ample chemotactic activity.[30] The mechanism by which monocytes adhere to mature crosslinked elastin but not to elastin fragments or elastin monomer is not yet known.

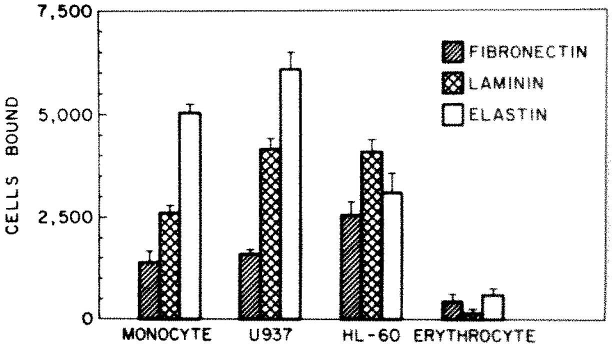

Fig 3. Comparative binding of human peripheral monocytes, U937 cells, HL60 cells, and erythrocytes to elastin, laminin, and fibronectin. Radiolabeled monocytes, U937 cells, HL60 cells, and human erythrocytes were incubated for 25 minutes at 37°C with nylon membranes containing 75 μg/dot elastin, laminin, or fibronectin. Background binding to untreated membranes was subtracted for each cell type. Data represent the number of cells bound per 4-mm dot (mean ± SD, n = 4).

Monocyte adhesion to the subendothelial basal lamina has been proposed to play a role in the pathogenesis of atherosclerosis.[1,2] These monocytes may be precursors of the lipid-laden foam cells characteristic of atherosclerotic lesions.[3,4] Both elastin and laminin are major components of this basal lamina and are well situated to mediate monocyte attachment. Laminin has also been shown to be chemotactic for tumor cells,[31] but its ability to mediate monocyte motility has not yet been determined. One question that remains to be answered is why the monocytes do not penetrate farther into the vascular wall, since they release enzymes that degrade basement membrane components and can migrate in response to those degradation products. Further studies on the regulation of adhesion, proteolytic enzyme production, and cell motility should provide new insights into monocyte participation in vascular disease processes.

ACKNOWLEDGMENT

We thank Dr Michael Bevilacqua, Brigham and Women's Hospital, Boston, MA for providing the human monocytelike cell lines and Dr Stuart Chipman, Children's Hospital, Boston, MA for advice and assistance in the preparation of tropoelastin monomer.

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CORRESPONDENCE

IMMUNE LYMPHOCYTE SURVIVAL AFTER CHEMOTHERAPY AND RADIATION

To the Editor:

This letter is in reply to a concise report recently published in *Blood.*[1] We would like to add our own evidence that immune lymphocytes survive after high-dose cyclophosphamide (60 mg/kg) body weight/day × 2 days), arabinosyl cytosine (3 g/m^2 body surface × 2, 12 hours apart), and total body irradiation (1,200 rad in 3 days). We analyzed the phenotype of cells removed by lymphocytapheresis in five patients just prior to infusion of donor marrow and found 55% $T3^+$, 45% $T4^+$, 22% $T8^+$, 27% $T10^+$, 17% Leu 11^+, 26% $M1^+$, and 4% $M3^+$ cells (mean values). To test the functional ability of these cells, we stimulated them with phytohemagglutinin and Daudi cells for 48 hours and then assayed for interleukin-2 (IL 2) activity. These cells, after high-dose chemotherapy and irradiation, produced 1.5 times the amount of IL 2 generated by untreated, normal lymphocytes in the same way. Similarly, we noted high IL 2 activity in lymphocytes of patients with head and neck cancer after irradiation.

Malkovsky and Medawar[2] propose that IL 2 is the determining factor in whether tolerance or T cell reactivity follows an immunological encounter. Antigen exposure in the absence of IL 2 results in tolerance. Our experience is in agreement with this. In 14 patients who were lymphocytapheresed just prior to donor marrow infusion (after pretransplant conditioning), graft *v* host disease (GVHD) developed in only 2 (14%) and a mild generalized maculopapular rash developed in only 1. Furthermore, if the natural killer (NK) cells we found in the host are functional and their activity is enhanced by IL 2 as suggested by Domzig and co-workers,[3] theoretically they could be responsible for the graft rejection that occurs in some patients.

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ANNOUNCEMENTS

DR H. DUBOIS-FERRIÈRE—DINU LIPATTI RESEARCH FELLOWSHIP IN HEMATOLOGY

The Dr H. Dubois-Ferrière—Dinu Lipatti Foundation offers a research fellowship to a medical graduate with at least two years' experience in research. The recipient of the fellowship will have the opportunity to spend two or three years working on a hematology research program in Geneva. The annual stipend ranges from US $30,000 to $35,000.

For further information, contact: Fondation Dr H. Dubois-Ferrière—Dinu Lipatti, case postale 225, CH-1227 Carouge-Genève, Switzerland.

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SECOND INTERNATIONAL CONFERENCE ON THALASSEMIA AND THE HEMOGLOBINOPATHIES

October 21-24, 1987
Herakleion, Crete, Greece

The Conference aims to unite the Third Mediterranean Meeting on Thalassemia and the respective meetings of the AMEDETH (*Association Mediterraneenne pour l'etude, le depistage et le traitement des hemoglobinoses*) and the *Club de l'hemoglobine* in an effort to avoid repetition, upgrade the level of presentations, promote collaboration, and save time and expenses. The scientific program will include lectures, round table discussions, and poster sessions covering the field of thalassemia, namely its molecular biology, therapeutic modalities, and evaluation, as well as prevention and social aspects. The Conference will be preceded by a training seminar. Crete is an ideal place for the Conference at this time of the year: the weather is pleasant and warm and the island is full of natural beauty and archaeological sites of utmost interest.

For further information, please write to the 2nd International Conference on Thalassemia and the Hemoglobinopathies, Secretariat, First Department of Medicine, University of Athens, PO Box 14 173, 115 10 Athens, Greece.

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FOURTH INTERNATIONAL WORKSHOP AND CONFERENCE ON HUMAN LEUKOCYTE DIFFERENTIATION ANTIGENS

1989
Vienna

The aim of this workshop series is to exchange antihuman leukocyte monoclonal antibodies among laboratories, to characterize and compare the nature of the antigens identified, to analyze the data centrally, and to publish the results jointly. The preparations for the wet workshop part, which will be followed by a joint conference in Vienna, are now beginning. The workshop will be organized into six sections: T cells, B cells, NK cells, myeloid cells, platelet antigens, and activation antigens.

Those who are interested in submitting monoclonal antibodies and/or receiving certain antibodies for in-depth evaluation are requested to write at their earliest convenience to: Fourth International Workshop on Human Leukocyte Differentiation Antigens, Attn. W. Knapp, Institute of Immunology, University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria.

AN OPEN INTERNATIONAL DISCUSSION SYMPOSIUM "NEWER STRATEGIES IN THE MANAGEMENT OF THROMBOTIC DISORDERS"

November 12-14, 1987
Loyola University Medical Center, Maywood, IL

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G.V.R. Born, *London, England*
J.P. Caen, *Paris, France*
J. Caprini, *Chicago, IL*
J. Choay, *Paris, France*
U. Cornelli, *Milan, Italy*
J.F. Davidson, *Glasgow, UK*
J. Fareed, *Chicago, IL*
M. Gent, *Hamilton, Ontario*
V. Gurewich, *Boston, MA*
F. Leja, *Chicago, IL*
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Topics: heparin and its derivatives; Ticlopidine—newer antiplatelet drugs; T-PA and related thrombolytic agents; Defibrotide and related drugs; viscosity modulators; biotechnology-based products; polytherapy of thrombotic disorders; mechanical devices; physical devices; and target-specific agents.

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INTERNATIONAL SOCIETY OF HEMATOLOGY SIXTH INTERNATIONAL CONGRESS OF THE ASIA-PACIFIC DIVISION

December 6-10, 1987

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ERRATA

In the article by Graf and Pesando entitled "Evidence for Chemical Differences in HLA-DR Molecules on Autologous Acute Lymphoblastic Leukemia and B-Lymphoblastoid Cell Lines," which appeared in the January 1987 issue (Volume 69, pp 7-11), the bottom of Figure 7 is not shown. Figure 7 is reprinted in its entirety below.

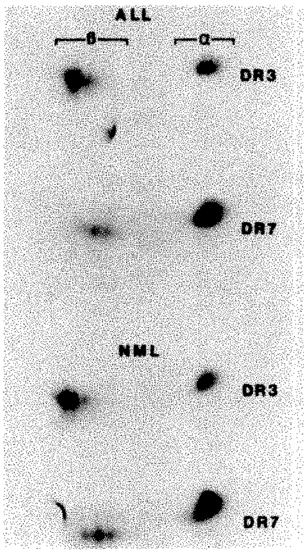


Fig 7. Two-dimensional gel electrophoresis of neuraminidase treated HLA-DR molecules from NALM-6 (ALL) and B85 (NML) cells. The HLA-DR3-specific MoAb I-2 and the HLA-DR7–specific MoAb SFR16-DR7M were used to study both of these cell lines. All four samples were compared simultaneously as in Fig 6.

In the article by Sorette et al entitled "Ektacytometric Measurement of Sickle Cell Deformability as a Continuous Function of Oxygen Tension," which appeared in the January 1987 issue (Volume 69, pp 316-323), the legends for Figures 3-6 were transposed and text citations of those Figures were misnumbered. The following two pages show Figures 3-6 with the correct legends and text citations. Address requests for corrected reprints to Margaret R. Clark, PhD, Box 0128, Cancer Research Institute, University of California, San Francisco, CA 94143.

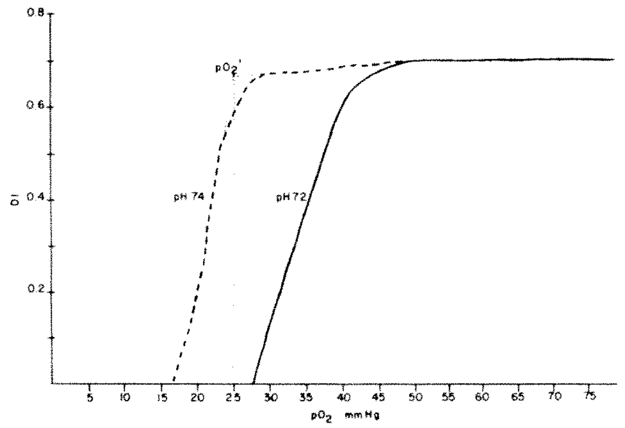


Fig 2. Variation in deformability signal (DI) as a function of oxygen tension for a Fraction 2 gradient population. As shown here, cell deformability decreased abruptly over a relatively narrow range in pO_2, producing a curve that essentially represented two intersecting lines. The point of intersection was used to define the pO_2 at which loss of deformability was first detected (pO_2'). Reduction of the pH from 7.4 to 7.2 shifted this critical pO_2 to higher values, reflecting the pH-induced increase in HbS polymerization.

down to a critical pO_2 level, after which it decreased in essentially linear fashion. Thus, the intersection of the two linear portions of the curve was used to identify the pO_2 value at which a loss of deformability was first detectable (designated pO_2' in Fig 2). In addition, because this value was expected to vary with MCHC, we also used the pO_2 at which the DI fell to a value of 0.2 to characterize each curve. The data shown in this figure and in Figs 4 through 6 were obtained on cell populations collected from the interface between the first and second layers of Stractan (Fraction 2). Such density-separated cell populations displayed a sharper transition in deformability with oxygen tension than whole blood, as expected from their greater homogeneity (data not shown). It can be seen that these Fraction 2 samples in the various experiments did not give identical DI *v* pO_2 curves. Comparison of the oxygen dissociation curves for the samples showed that differences in oxygen affinity, presumably due to individual patient differences and differences in cell storage time, were the major source of variation.

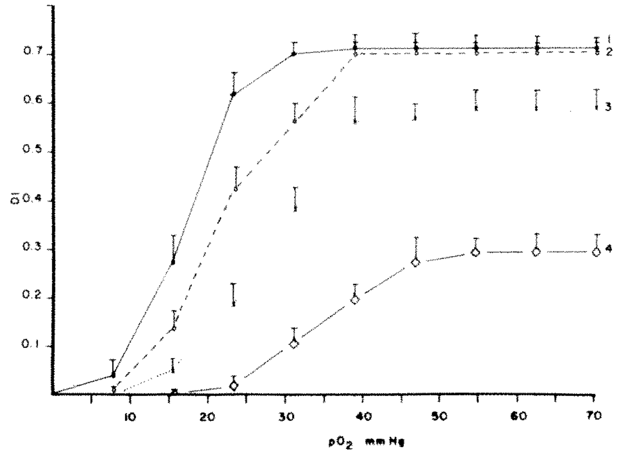


Fig 3. DI *v* pO_2 curves for Fractions 1 to 4 of Stractan-separated sickle cells. As the MCHC of isolated cell populations increased, the maximum DI decreased, and a deoxygenation-dependent loss in cell deformability occurred at progressively higher pO_2. See Table I for characteristics of the cell populations.

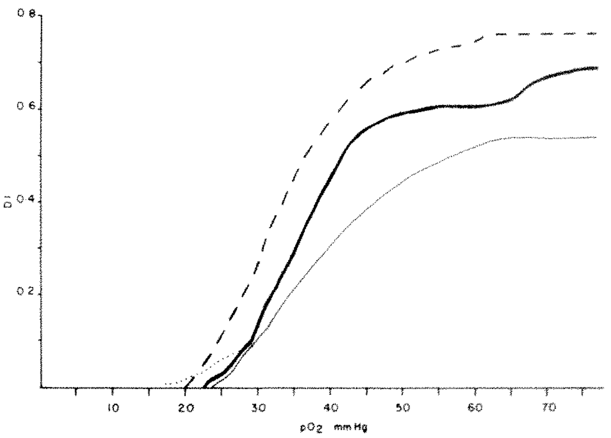


Fig 4. Effect of suspending medium viscosity on the DI *v* pO_2 curve for a Fraction 2 gradient population. A decrease in viscosity below 19.5 cp at 37 °C cp (4% PVP concentration) resulted in overall reduction in cell deformation and eventual blurring of the deoxygenation-dependent loss of cell deformability. (-----) 19.5 cp, (——) 12.5 cp, (——) 9.5 cp, (·····) 7.5 cp.

A series of experiments was performed to determine the effect of important experimental variables and to define the optimal procedure to obtain reproducible, physiologically relevant data. As expected from the characteristics of the polymer equilibrium, several factors predictably affected the range of oxygen tension over which the cell deformability loss occurred. Reduction of the suspending medium pH from 7.4 to 7.2 caused a parallel shift of the DI *v* pO_2 curve to higher pO_2 (Fig 2), consistent with the increased Bohr effect of HbS, particularly below an extracellular pH of 7.4, and the corresponding enhancement of polymer formation.[4,20] Most of the experiments employed paired deformability and oxy-

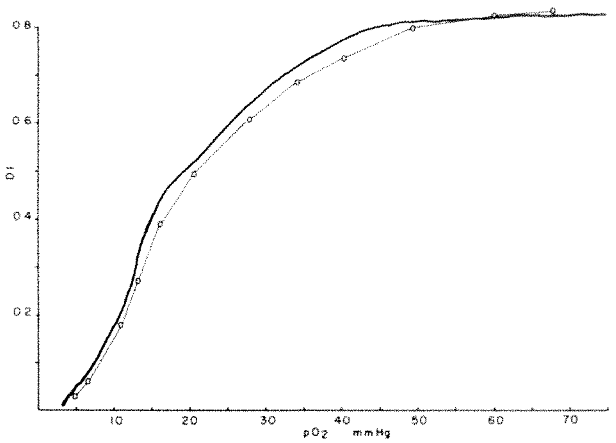


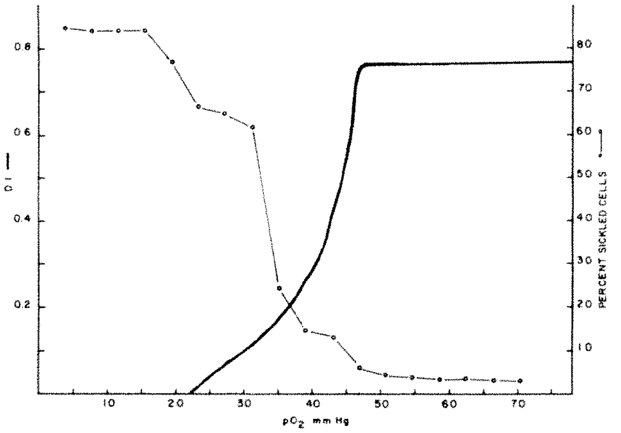
Fig 5. Comparison of DI *v* pO_2 relationship for continuous pO_2 gradient (continuous curve) and for measurements performed at fixed pO_2 values (open circles) over the same range for a Fraction 2 gradient population.

gen saturation measurements at both pH 7.2 and 7.4 to permit comparison of the results with those of Noguchi et al[7] and Noguchi[17] on polymer content, since those experiments were performed at pH 7.2. Some additional experiments were performed only at pH 7.4.

The position of the DI *v* pO_2 curve also varied with MCHC, as shown by measurements using subpopulations of sickle cells isolated on density gradients (Fig 3). Again, the shift of the curves to higher pO_2 with increasing MCHC was consistent with the known increase in polymer content with increasing MCHC for any given oxygen tension.[4,21] The DI versus pO_2 curves for unseparated cells (data not shown) were always broader than those for the gradient populations, reflecting the fact that the heterogeneous mixture of cells underwent polymerization and sickling over a broad pO_2 range.

Another variable that influenced the position of the DI *v* pO_2 curve was the viscosity of the suspending medium. A decrease in extracellular viscosity, achieved by reducing the concentration of PVP, resulted in a reduction in the proportion of deformable cells at progressively higher pO_2 values. For higher MCHC cells there was also a reduction in the maximum attainable DI (Fig 4). We attribute this latter effect to the requirement for a threshold ratio of extracellular to intracellular viscosity to induce red cell deformation.[22] A viscosity of 19.5 cp (4% PVP at 37 °C) was chosen for subsequent experiments, since this viscosity showed clear-cut deformability differences among normal red cells from different density gradient subpopulations without introducing large variations among whole blood samples from different normal subjects. It was found that if the viscosity was reduced much below 19 cp, a substantial and varying proportion of cells from normal individuals would not deform, leading to a broadening of the range of normal isotonic DI values.

Other experimental variables tested for their effect on the deformability-oxygen tension relationship included the cation composition of the suspending medium, the flow rate of the cell suspension through the gas exchange fibers, and the time period between gas exchange and deformability measurement. When cells were equilibrated in high-Na, low-K medium, the DI-pO_2 curve was shifted to higher oxygen tensions as compared to the curve obtained for cells from the same sample that were equilibrated in a medium in which the Na and K concentrations were close to their intracellular concentrations. This difference was interpreted as a reflection of a net leakage of K ions out of sickled cells in the high Na medium, resulting in a loss in cell water and a concomitant increase in HbS concentration and polymer content. Therefore the buffer that minimized transmembrane cation gradients was used for the rest of the experiments.



Relationship between the reduction in the DI signal and percentage of sickle cells for a Fraction 2 gradient population.

Table 1. Characteristics of Sickle Cell Gradient Subpopulations

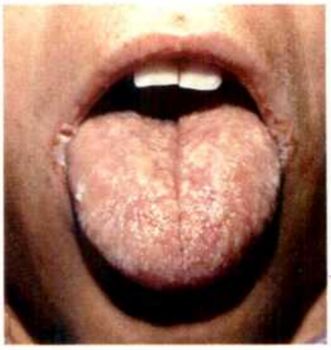
Fraction	MCHC (g/dL)	Reticulocytes (%) ($\bar{X}$ ± SEM)	ISC (%)
1	26.00 ± 0.72	10.95 ± 1.14	2.12 ± 0.29
2	29.49 ± 0.44	7.84 ± 1.09	2.95 ± 0.48
3	31.21 ± 0.47	5.59 ± 0.55	3.82 ± 0.46
4	33.20 ± 0.47	4.22 ± 0.55	5.95 ± 0.60
5	34.75 ± 0.37	2.83 ± 0.80	7.58 ± 0.93
6	39.48 ± 0.91	1.40 ± 0.19	12.59 ± 1.08

The rate at which the cells were pumped through the gas exchange fibers affected the subsequent deformability of even fully oxygenated cells. If the flow rate was high (>4 mL/min), the maximum cell deformation was decreased. We surmise that this was likely the result of shear-induced fragmentation occurring as the cells entered or left the fibers. Calculation of Reynolds numbers for flow through the fibers at these rates indicates that turbulence was probably present, increasing the likelihood of cellular fragmentation. The delay time for polymer formation at low HbS concentration and at pH 7.4 was expected to be prolonged, particularly at higher levels of oxygen saturation.[23] Therefore, we wanted to determine the maximum period for which the cells could be equilibrated at 37 °C without undergoing damage and alteration in their deformability properties that could not be explained on the basis of HbS polymer formation. Variation of the equilibration period from 30 to 90 minutes had no significant effect on the DI *v* pO_2 curve (data not shown). An equilibration period of 30 minutes was chosen for the study.

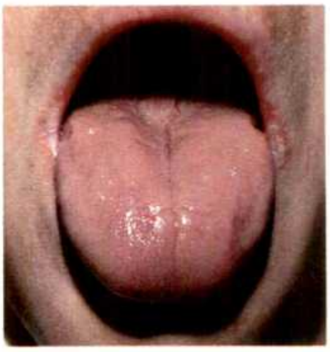
We performed experiments in which 10-mL aliquots of cell suspension were run through the system while a constant pO_2 was maintained in the gas exchange system (Fig 5). Measurement of the DI at various fixed pO_2 values along the gradient range showed that the DI-pO_2 relationship obtained was very close to the continuous curve obtained using the decreasing oxygen gradient (when the continuous curve was adjusted for the spatial separation of the oxygen electrode and the ektacytometer). Therefore, we conclude that measurements performed using a decreasing gradient from 76 to 0 mm Hg oxygen provide accurate information about the proportion of sickle cells that can deform after equilibration at a given pO_2 value.

Examination of cell morphology at various points along the pO_2 gradient allowed us to define the relationship between the reduction in the DI signal and the percentage of sickle cells in a representative isolated cell population with an MCHC of 32 g/dL at pH 7.2 (Fig 6). We observed both characteristic elongated cells with spicules, and a larger

The first troche treatment for oropharyngeal candidiasis.



Before treatment.[1]



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*Clinical cure is defined as absence of signs and symptoms of the disease.

†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

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Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

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Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

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Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

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How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: **1.** Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. **2.** Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **3.** Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. **4.** Data on file, Miles Pharmaceuticals.

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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

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6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

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7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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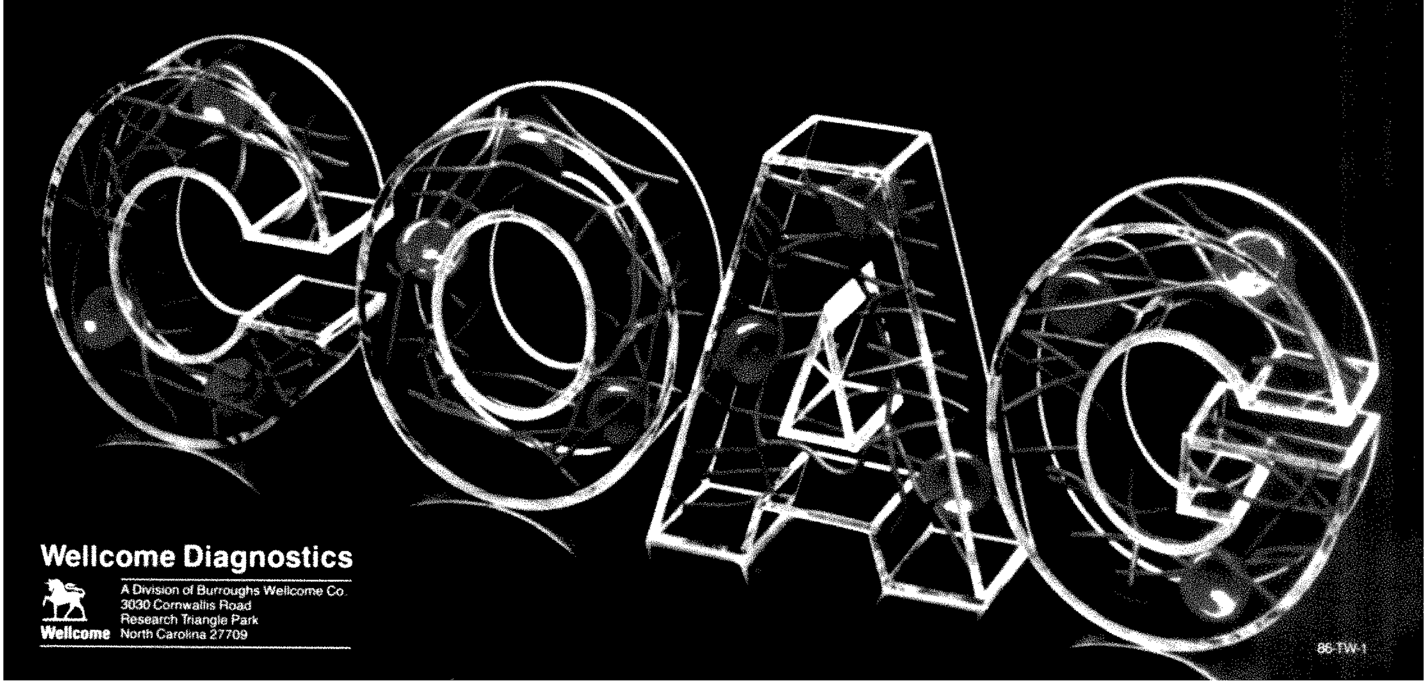

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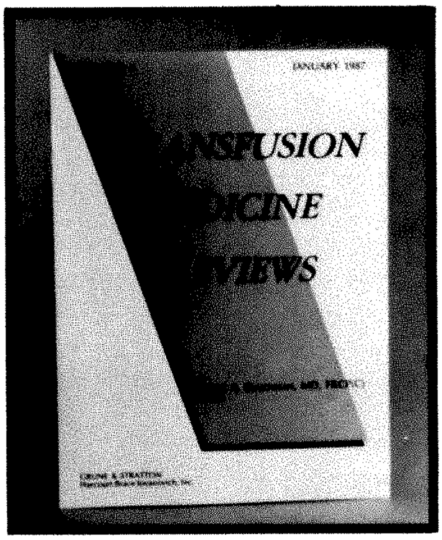

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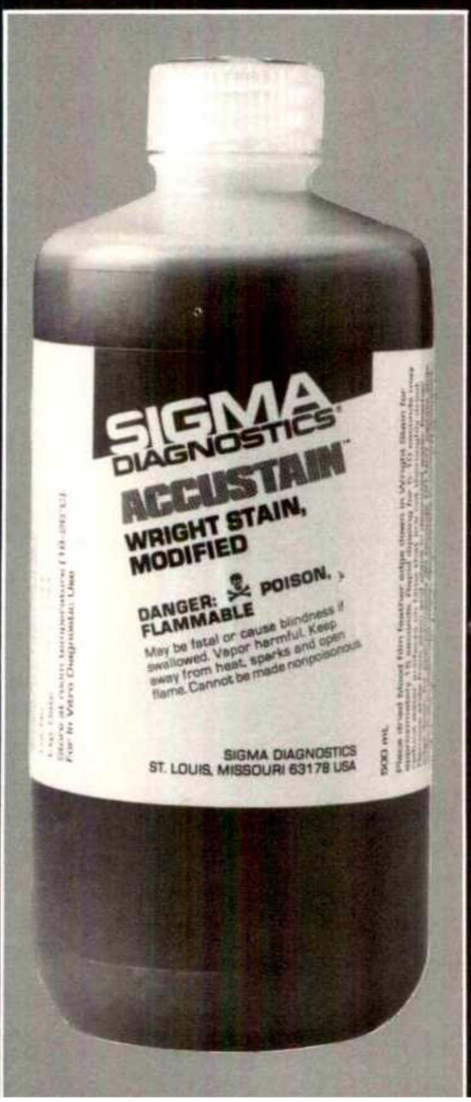
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DIRECTION INSERT
Immune Globulin Intravenous (Human)
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DESCRIPTION

Immune Globulin Intravenous (Human), GAMMAGARD® * is a sterile, dried, highly purified preparation of immunoglobulin which is derived from the cold ethanol fractionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with the appropriate volume of diluent, this preparation contains approximately 50 mg of protein per mL, of which at least 90% is gamma globulin. The reconstituted product contains approximately 1% sodium chloride, not more than 20 mg/mL glucose, not more than 0.2 g/dL PEG, and 0.3M glycine as a stabilizing agent. It has a pH of 6.8±0.4.

The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average, the distribution of IgG subclasses present in this product is the same as is present in normal plasma.[1] Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD contains no preservative.

This product has been prepared from large pools of human plasma which was taken only from plasma units found to have normal levels of alanine aminotransferase (ALT). Each unit of plasma used in the manufacture of this product has been found to be nonreactive for HBsAg and HTLV-III antibody by FDA approved tests.

CLINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum levels is, therefore, to be expected.[2]

As a class, IgG survives longer *in vivo* than other serum proteins.[2,3] Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.[2,3,4] The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.[2,3,4,5]

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.[4,5]

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 μg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea may occasionally occur. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring clinical response and/or the serum IgG levels before each dose, to insure that they do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg per Hr be used. If infusion at this rate causes the patient no distress, the administration rate may be gradually increased but should not exceed 4 mL/kg per Hr.

A rate of administration which is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGARD should be administered as soon after reconstitution as possible. Administration should not begin more than 2 hours after reconstitution.

The reconstituted material should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure the administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGARD is supplied in either 2.5 g or 5.0 g single use vials. Each vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human), GAMMAGARD should be stored under ordinary refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing should be avoided to prevent the diluent bottle from breaking.

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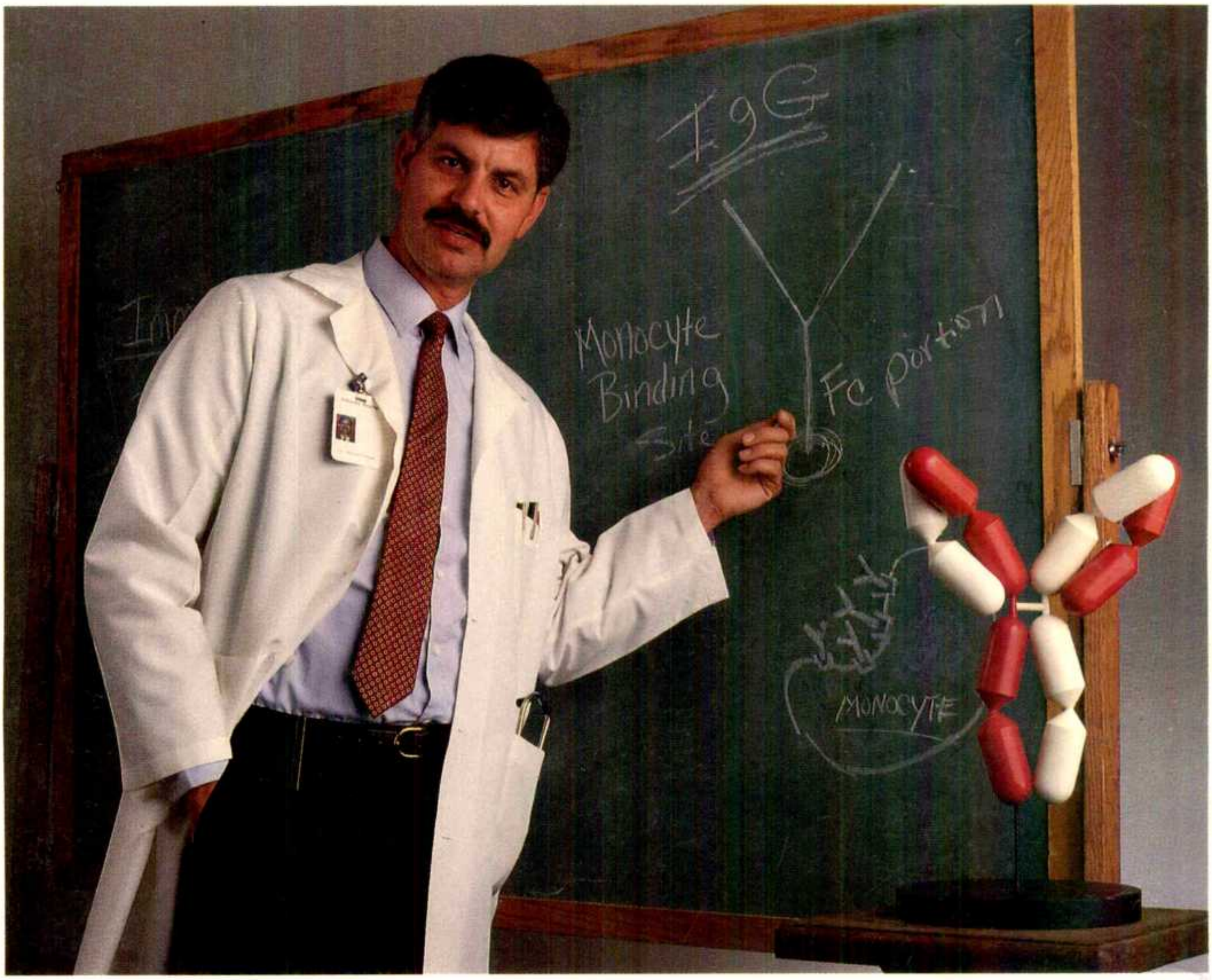

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[1] Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.

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Before prescribing WELLCOVORIN® Tablets, please consult complete prescribing information. The following is a brief summary.

INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

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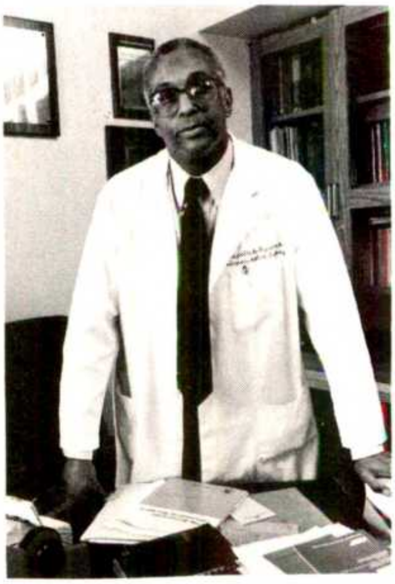

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The warning signs for colorectal cancer are a change in bowel habits and blood in the stool.

People with a family history of colon or rectal cancer or ulcerative colitis are at higher risk and are urged to be doubly cautious.

Checkup Guidelines for men and women over 50 without symptoms:

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- stool blood test annually
- procto exam every 3 to 5 years after 2 negative tests 1 year apart.

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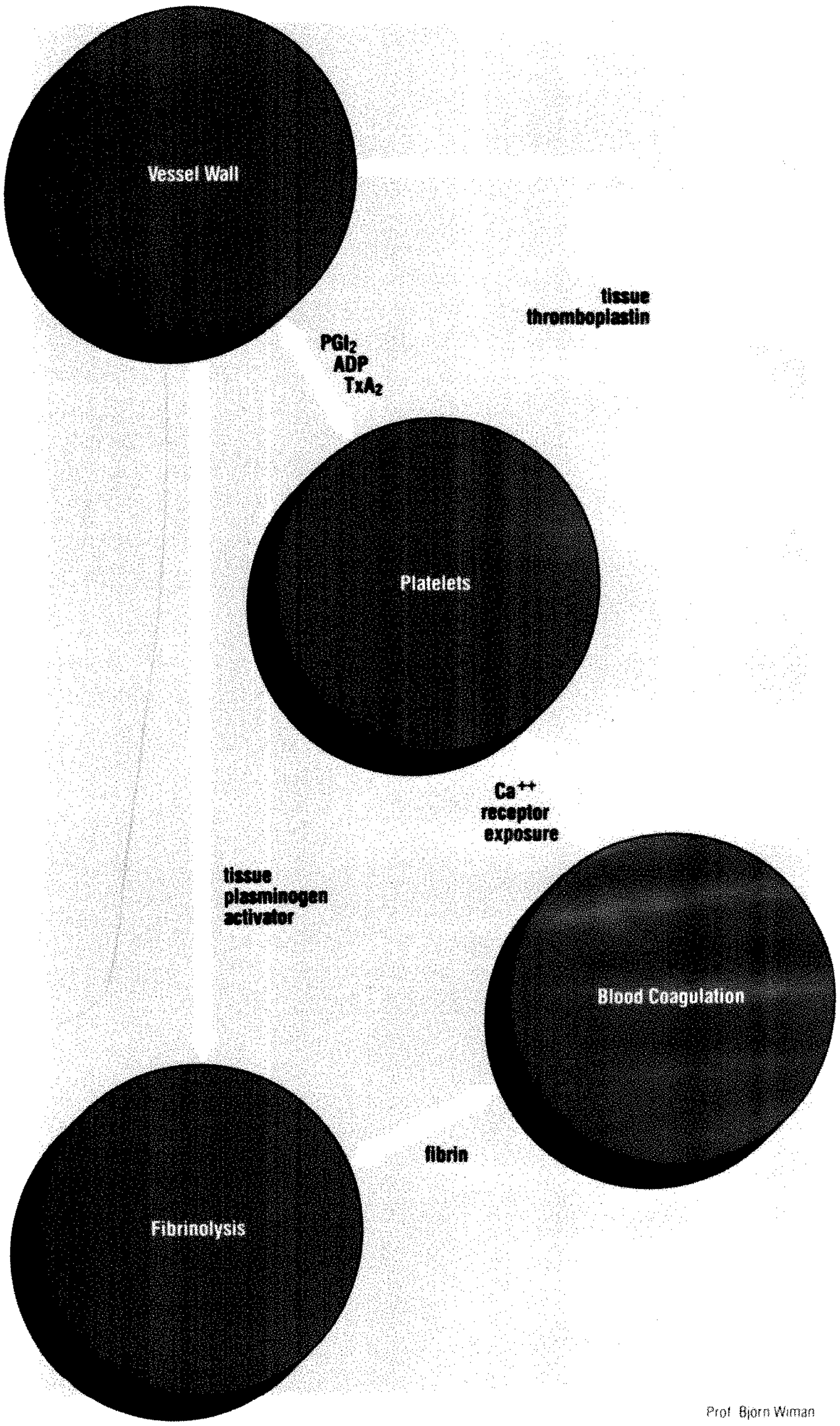

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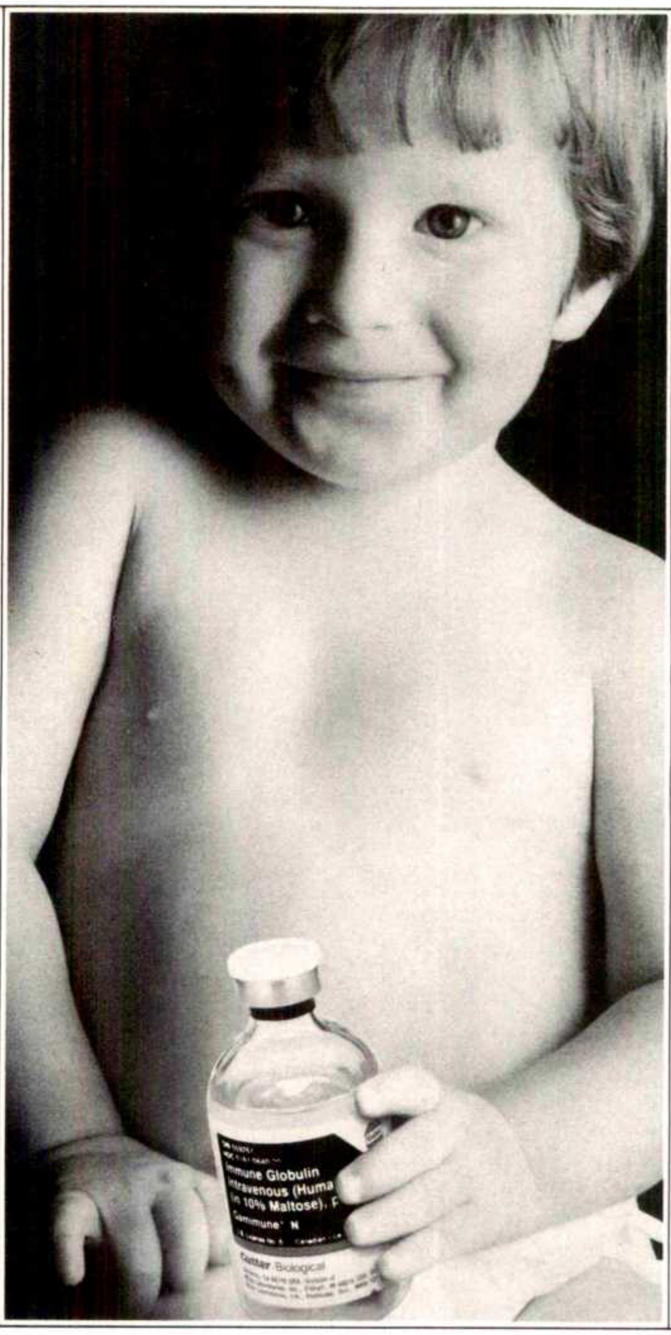

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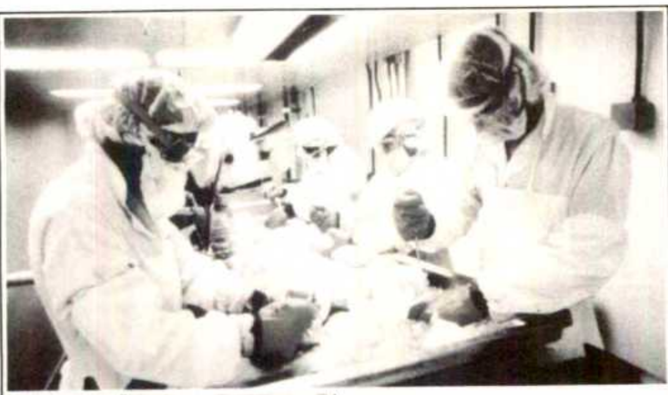

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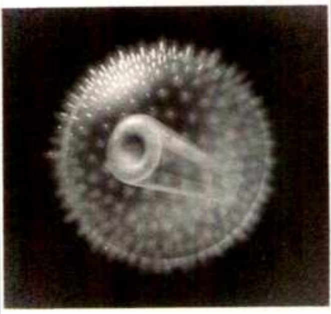

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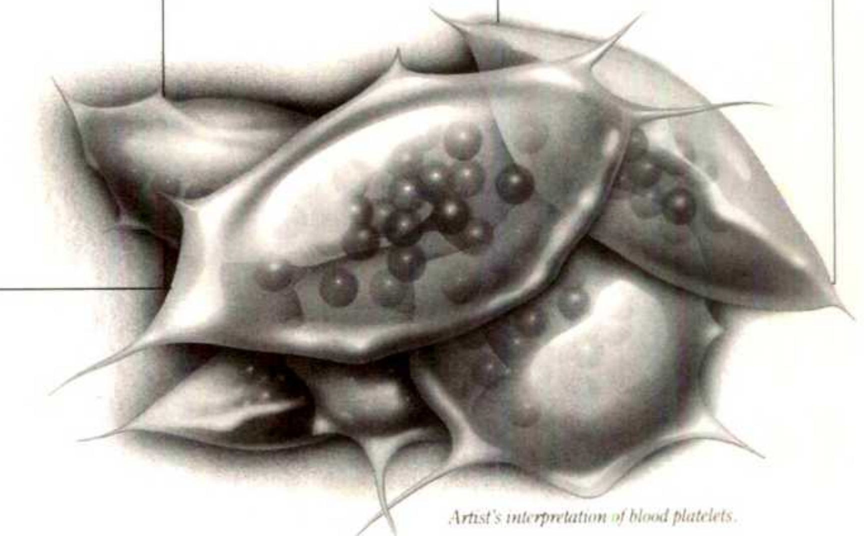

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[*] It is not possible to predict which patients with ITP will respond to therapy.

[†] In patients who respond to therapy, a rise in platelet count is generally rapid (within 1 to 5 days), transient (most often lasting from several days to several weeks) and should not be considered curative.

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CLINICAL PHARMACOLOGY

Gamimune* N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune* N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune* N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune* N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune* N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune* N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune* N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune* N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune* N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune* N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune* N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune* N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune* N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune* N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune* N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune* N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune* N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune* N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune* N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune* N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune* N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune* N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune* N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune* N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune* N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune* N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune* N. It is not known whether Gamimune* N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune* N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune* N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune* N. Symptoms reported with Gamimune* N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune* N at the infusion site was reported in some cases.

In further studies of Gamimune* N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune* N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune* N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune* N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune* N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune* N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune* N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune* N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune* N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune* N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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REFERENCES: 1. GAMIMUNE® N Product License Application. **2.** Lundblad JL, Mitra G, Steinberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. Rev Infect Dis 1986;8(Jul–Aug): S382–S390. **3.** Mitra G, Wong MF, Mozen MM, et al: Elimination of infectious retroviruses during preparation of immunoglobulins. Transfusion 1986;26(4):394–397.

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2. In front of a mirror. Observe breasts. Arms at sides. Raise arms high overhead. Any change in nipples, contours, swelling, dimpling of skin? Palms on hips: press down firmly to flex chest muscles. Breasts do not usually match.

3. Lying down. Pillow under right shoulder, right hand behind head. Left hand fingers flat, press gently in small circular motions starting at 12 o'clock. Make about three circles moving closer to and including nipple. Reverse and repeat on left.

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Announcing

RETROVIR® (zidovudine)

The first effective treatment for certain AIDS and other serious HIV* infections

AZT™ (azidothymidine)
IS NOW
RETROVIR® (zidovudine)

---

*Human immunodeficiency virus

Please see brief summary of prescribing information on last page of this advertisement.

RETROVIR® (zidovudine)

New hope for many AIDS/ARC patients

A multicenter study proved…

RETROVIR can prolong the lives of certain AIDS and ARC patients

- In a double-blind placebo-controlled clinical trial (N = 281), only one death occurred among 144 RETROVIR patients *v* 19 deaths of the 137 patients on placebo ($p<0.001$). The study population consisted of AIDS patients within 120 days of their first diagnosis of *Pneumocystis carinii* pneumonia or patients with more severe manifestations of AIDS-related complex (ARC)
- The probability of 24-week survival for any patient in the RETROVIR group was 0.98 compared to 0.78 for the placebo group

Comparative Mortality of RETROVIR and Placebo Group Patients in Controlled Clinical Trial (stratified by diagnosis and T4 count) n = 281

Stratum	Group Assigned	Number of Deaths
AIDS (n=160)	RETROVIR	1
	PLACEBO	12
ARC (n=121)	RETROVIR	0
	PLACEBO	7

Stratum	Group Assigned	Number of Deaths
Higher T4 count ($\geq$100 $\leq$500) (n=100)	RETROVIR	0
	PLACEBO	4
Lower T4 count (<100) (n=181)	RETROVIR	1
	PLACEBO	15

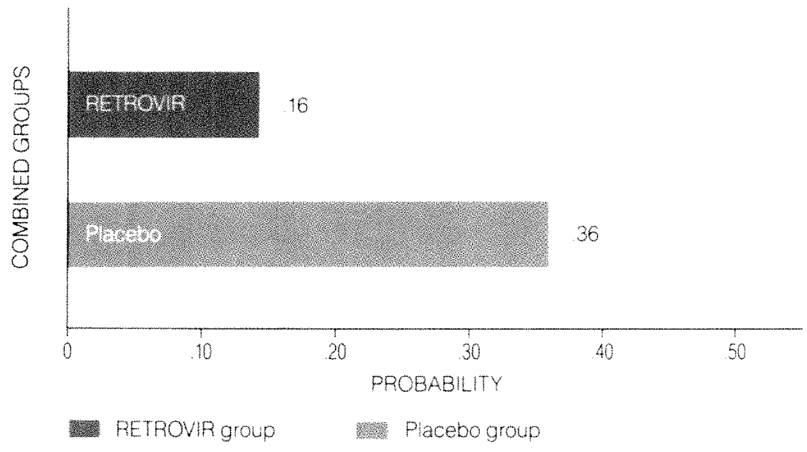
Total Deaths	RETROVIR	1
	PLACEBO	19

RETROVIR® (zidovudine) reduced the risk and severity of opportunistic infections

- RETROVIR significantly reduced the probability of acquiring opportunistic infections in AIDS and ARC patients ($p<0.001$)
- Severity of opportunistic infections was significantly reduced in RETROVIR patients as compared with controls ($p<0.005$)
- In the controlled trial, no opportunistic infections occurred in ARC patients who had received at least six weeks of RETROVIR therapy. Some ARC patients developed opportunistic infections in the follow-up period while on drug

Probability of Developing Opportunistic Infection Within 24 Weeks

(Excludes opportunistic infections acquired within six weeks of entry into the study)



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WARNING

WARNING: Therapy with RETROVIR® (zidovudine) is often associated with hematologic toxicity including granulocytopenia and severe anemia requiring transfusions (see WARNINGS). In addition, patients treated with zidovudine may continue to develop opportunistic infections (OI's) and other complications of the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) caused by the human immunodeficiency virus (HIV). Therefore, patients on zidovudine should be under close clinical observation by physicians experienced in the treatment of patients with diseases associated with HIV. The safety and efficacy of zidovudine have been established only for certain adult AIDS and advanced ARC patients (see INDICATIONS AND USAGE).

IMPROVING LIVES THROUGH
ANTIVIRAL RESEARCH

BURROUGHS WELLCOME CO.

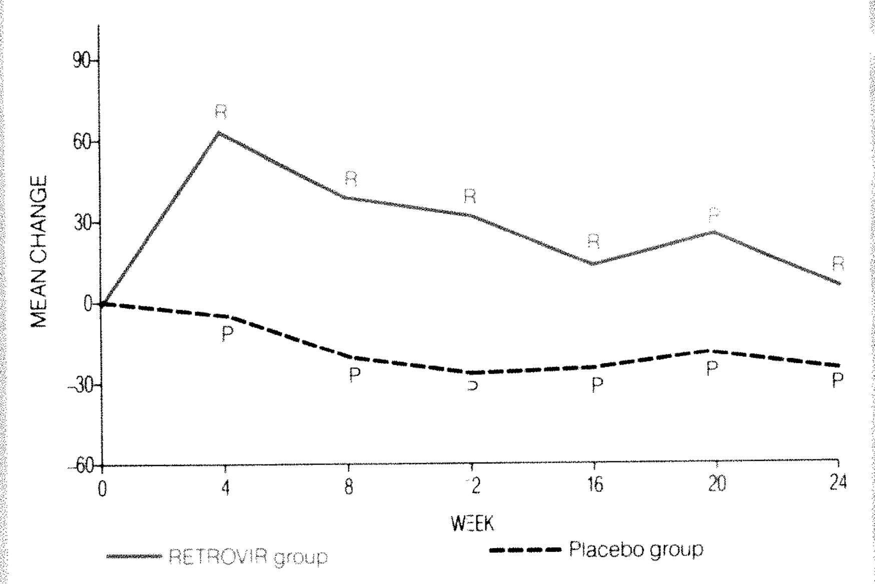
RETROVIR® (zidovudine)

New hope for many AIDS/ARC patients

RETROVIR improved immune status

- T4 cell counts in the RETROVIR group remained significantly higher than in the control group throughout the study
  - In some RETROVIR patients, particularly those with lower T4 cell counts at entry, T4 cell numbers increased, then subsequently declined
  - In contrast, placebo-group patients experienced a progressive decline from baseline in number of T4 cells
- The number of positive delayed hypersensitivity skin test conversions was significantly higher in the RETROVIR group ($p<0.001$)

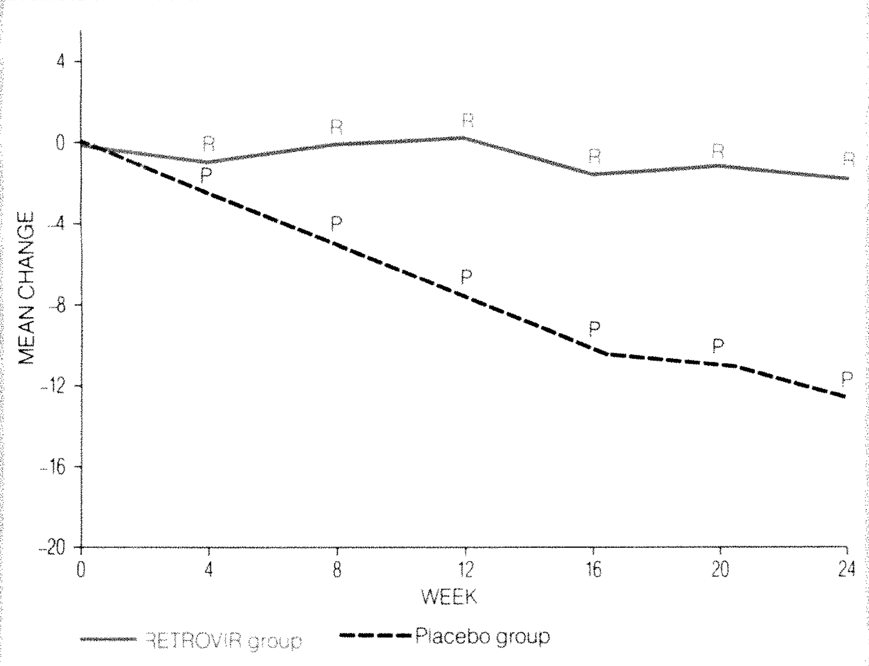
Change in T4 Cell Count



Patients' general health improved

- Patients on RETROVIR® (zidovudine) maintained their high Karnofsky performance scores (an index measuring ability to perform activities of daily life), whereas placebo group patients' scores declined
- RETROVIR patients experienced significantly fewer symptoms related to HIV infection than those on placebo
- Patients on RETROVIR therapy maintained their body weight throughout the study, whereas placebo group patients progressively lost weight

Change in Karnofsky Score for All AIDS/ARC Patients Combined



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IMPROVING LIVES THROUGH
ANTIVIRAL RESEARCH

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RETROVIR® (zidovudine)

New hope
for many AIDS/ARC patients

Clinical studies showed...

It is important to identify patients at greatest risk for bone marrow toxicity, and subsequent RBC and WBC suppression, and monitor carefully

- 25% of RETROVIR patients developed anemia (<7.5 g/dl) as compared to 4% in the placebo group. This adverse reaction was most likely to occur in AIDS patients and patients with lower numbers of T4 cells
- 31% of RETROVIR patients required at least one transfusion, compared with 11% of placebo patients
- Granulocyte depression (<750/mm^3) was observed in 39% of RETROVIR patients as compared with 9% of placebo patients. Granulocytopenia occurred more frequently in AIDS patients and patients with lower T4 cell counts

Clinical adverse events

- Although the majority of patients in *both* the RETROVIR (84%) and control groups (72%) reported the occurrence of at least one adverse event, nausea, myalgia, insomnia, and moderate-to-severe headache were significantly more frequent among RETROVIR patients

The potential hematologic toxicity of RETROVIR plays an important role in patient management

- The majority of patients who required adjustment of RETROVIR dosage had hematologic toxicity, specifically anemia or granulocytopenia, usually occurring within the first four to eight weeks of RETROVIR therapy
- Because of this potential, careful monitoring of hematologic indices is recommended every two weeks*

Drug interactions must be considered with RETROVIR

- Concomitant use of acetaminophen with RETROVIR may potentiate granulocytopenia; other drugs that may interfere with RETROVIR metabolism or excretion (eg, probenecid, lorazepam, indomethacin, cimetidine) should be avoided

RETROVIR—the newest antiviral breakthrough from Burroughs Wellcome Co.—is the first effective treatment for certain HIV infections.

---

*RETROVIR dose interruption may be required for significant anemia or granulocytopenia until evidence of bone marrow recovery is observed. A reduction in daily dose may be adequate for less severe depression of RBC/WBC counts.

Since the initial supply of RETROVIR® (zidovudine) is limited, a special distribution program is in place

- Rapidity of development and complexity of the production process have limited the current supply of RETROVIR
- To help ensure a continuous supply of RETROVIR for patients at greatest need and those who would benefit most from treatment during this period of limited supply, a special distribution program has been established
- An independent group of experts from the Infectious Disease Society of America (IDSA) has assisted in developing this program for the best utilization of RETROVIR

Patients can be enrolled in the RETROVIR distribution program now

- You can apply to enroll your patients in the RETROVIR distribution program at this time
- If you are currently treating AIDS or ARC patients and need additional information, call 1-800-843-9388
- Detailed information and enrollment applications are also available from your Burroughs Wellcome Co. Sales Representative
- Only patients enrolled in the distribution program will be able to receive RETROVIR

Burroughs Wellcome Co.: Leader in Antiviral Research

- Continuing clinical trials to evaluate further the role of RETROVIR in the treatment of HIV infections
- Conducting research for antiviral agents effective against varicella-zoster, Epstein-Barr, human papilloma, hepatitis B virus, and cytomegalovirus
- Providing physicians with antiviral products effective against herpes simplex — today's hope for patients with genital herpes
- **Pledging to keep you updated as new information becomes available**

If you have patients with HIV infections and need additional information, please call

1-800-843-9388

IMPROVING LIVES THROUGH
ANTIVIRAL RESEARCH

BURROUGHS WELLCOME CO.

Please see brief summary of prescribing information on last page of this advertisement.

RETROVIR® (ZIDOVUDINE) Capsules

WARNING: THERAPY WITH RETROVIR (ZIDOVUDINE) IS OFTEN ASSOCIATED WITH HEMATOLOGIC TOXICITY INCLUDING GRANULOCYTOPENIA AND SEVERE ANEMIA REQUIRING TRANSFUSIONS (SEE WARNINGS).

IN ADDITION, PATIENTS TREATED WITH ZIDOVUDINE MAY CONTINUE TO DEVELOP OPPORTUNISTIC INFECTIONS (OI'S) AND OTHER COMPLICATIONS OF THE ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) AND AIDS RELATED COMPLEX (ARC) CAUSED BY THE HUMAN IMMUNODEFICIENCY VIRUS (HIV). THEREFORE, PATIENTS ON ZIDOVUDINE SHOULD BE UNDER CLOSE CLINICAL OBSERVATION BY PHYSICIANS EXPERIENCED IN THE TREATMENT OF PATIENTS WITH DISEASES ASSOCIATED WITH HIV. THE SAFETY AND EFFICACY OF ZIDOVUDINE HAVE BEEN ESTABLISHED ONLY FOR CERTAIN ADULT AIDS AND ADVANCED ARC PATIENTS (SEE "INDICATIONS AND USAGE").

INDICATIONS AND USAGE: Retrovir Capsules are indicated for the management of certain adult patients with symptomatic HIV infection (AIDS and advanced ARC) who have a history of cytologically confirmed *Pneumocystis carinii* pneumonia (PCP) or an absolute CD4 (T4 helper/inducer) lymphocyte count of less than $200/mm^3$ in the peripheral blood before therapy is begun.

This indication is based primarily on the results of a randomized, double-blind, placebo-controlled trial conducted at 12 medical centers in the United States in which 281 patients with AIDS or advanced ARC were studied for an average of four and a half months. Additional data have been collected on approximately 80% of these patients who have received zidovudine in an open-label extension of this trial for an average of five more months.

The patient population of the controlled trial consisted of 160 AIDS patients (85 Retrovir and 75 placebo) who had recovered from their first episode of PCP diagnosed within the previous four months, and 121 ARC patients (59 Retrovir and 62 placebo) with multiple signs and symptoms of HIV infection, including mucocutaneous candidiasis and/or unexplained weight loss ($\geq$ 15 lbs or $>$ 10% of prior body weight). All patients had evidence of impaired cellular immunity with an absence of delayed cutaneous hypersensitivity and a decreased number of CD4 (T4) lymphocytes in the peripheral circulation. Two hundred twenty-one (79% of all patients) had fewer than 200 T4 $cells/mm^3$ at entry (95% of AIDS patients and 57% of ARC patients). All patients began therapy at a dose of 250 mg every 4 hours around the clock. This dosage was reduced or temporarily or permanently discontinued if serious marrow toxicity occurred. The trial was stopped because of a significant reduction in mortality before all patients had completed the planned 24 weeks of treatment. There were 19 deaths in the placebo group and 1 in the Retrovir group ($p < .001$). All deaths were apparently due to opportunistic infections (OI) or other complications of HIV infection. Treatment duration ranged from 12 weeks to 26 weeks, with a mean and median duration of 17 and 18 weeks, respectively.

Retrovir also significantly reduced the risk of acquiring an AIDS-defining OI in patients after 6 weeks of treatment ($p < .001$). In addition, patients who received Retrovir generally did better than the placebo group in terms of several other measures of efficacy including performance level, neuropsychiatric function, maintenance of body weight and the number and severity of symptoms associated with HIV infection. A modest increase in mean CD4 (T4) counts was seen in the zidovudine group but the significance of this finding is unclear as the CD4 (T4) counts declined again in some patients.

The most significant adverse reaction noted in the study was a depression of formed elements in the peripheral blood, which necessitated dose reduction or drug discontinuation in 49 of the 144 (34%) patients receiving Retrovir. Of those participants whose baseline CD4 (T4) lymphocyte counts were less than 200, 47% of those receiving Retrovir and 10% of those receiving placebo developed a granulocyte count of $<750/mm^3$. Similarly, 45% of Retrovir recipients and only 14% of placebo recipients had a 25% or greater reduction in hemoglobin. (See ADVERSE REACTIONS.)

At the conclusion of the placebo-controlled trial, 127 Retrovir recipients and 100 placebo recipients elected to enroll in an uncontrolled extension protocol in which all patients received Retrovir at a dose of 200 mg every four hours. This dose was chosen because of concern about cumulative hematologic toxicity and to allow for greater flexibility in dosing. Over the subsequent five months, opportunistic infections continued to occur and additional patients died in both groups. Sixteen patients from the original placebo cohort died after the conclusion of the controlled trial. Four of these 16 never received Retrovir and seven expired during the first month of therapy. Ten additional deaths occurred among the original Retrovir recipients. With respect to these data, termination of the controlled trial precludes direct comparisons of mortality and morbidity between the original placebo and drug recipients. Long-term follow-up of these patients, however, will allow for examination of the natural history of prolonged zidovudine therapy.

CONTRAINDICATIONS: Retrovir Capsules are contraindicated for patients who have potentially life-threatening allergic reactions to any of the components of the formulation.

WARNINGS: Zidovudine has been carefully studied in limited numbers of seriously ill HIV-infected patients treated for a limited period of time. Therefore, the full safety and efficacy profile of zidovudine has not been completely defined, particularly in regard to prolonged use, and especially in HIV-infected individuals who have less advanced disease.

Zidovudine should be used with extreme caution in patients who have bone marrow compromise evidenced by granulocyte count $<$ $1000/mm^3$ or hemoglobin $<$ 9.5 g/dl. In the placebo-controlled study, anemia and granulocytopenia were the most significant adverse events observed (see ADVERSE REACTIONS).

Significant anemia most commonly occurred after 4 to 6 weeks of therapy and in many cases required dose adjustment, discontinuation of zidovudine, and/or blood transfusions. Frequent (at least every 2 weeks) blood counts are strongly recommended in patients taking zidovudine. If anemia or granulocytopenia develops, dosage adjustments may be necessary (see DOSAGE AND ADMINISTRATION).

Coadministration of zidovudine with other drugs metabolized by glucuronidation should be avoided because the toxicity of either drug may be potentiated (see Drug Interactions under PRECAUTIONS). Zidovudine recipients who used acetaminophen during the controlled trial had an increased incidence of granulocytopenia which appeared to be correlated with the duration of acetaminophen use.

PRECAUTIONS:

General: Zidovudine is eliminated from the body primarily by renal excretion following metabolism in the liver (glucuronidation). There are currently no data available concerning the use of zidovudine in patients with impaired renal or hepatic function, and such patients may be at a greater risk of toxicity from zidovudine.

Drug Interactions: The interaction of other drugs with zidovudine has not been studied in a systematic manner. Coadministration of zidovudine with drugs that are nephrotoxic, cytotoxic, or which interfere with RBC/WBC number or function (e.g., dapsone, pentamidine, amphotericin B, flucytosine, vincristine, vinblastine, adriamycin, or interferon) may increase the risk of toxicity. Limited data suggest that probenecid may inhibit glucuronidation and/or reduce renal excretion of zidovudine. In addition, other drugs (e.g., acetaminophen, aspirin, or indomethacin) may competitively inhibit glucuronidation (see WARNINGS).

Some experimental nucleoside analogues which are being evaluated in AIDS and ARC patients may affect RBC/WBC number or function and may increase the potential for hematologic toxicity of zidovudine. Some experimental nucleoside analogues affecting DNA replication antagonize the *in vitro* antiviral activity of zidovudine against HIV and thus, concomitant use of such drugs should be avoided.

Some drugs such as trimethoprim-sulfamethoxazole, pyrimethamine, and acyclovir may be necessary for the management or prevention of opportunistic infections. In the controlled trial, increased toxicity was not detected with limited exposure to these drugs. However, there are two published reports of neurotoxicity (one of profound lethargy and one of seizure) associated with concomitant use of zidovudine and acyclovir.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Long-term carcinogenicity studies of zidovudine in animals have not been completed. However, in an *in vitro* mammalian cell transformation assay, zidovudine was positive at concentrations of 0.5 μg/ml and higher.

No evidence of mutagenicity (with or without metabolic activation) was observed in the Ames *Salmonella* mutagenicity assay. In a mutagenicity assay conducted in L5178Y/$TK^{+/-}$ mouse lymphoma cells, zidovudine was weakly mutagenic in the absence of metabolic activation only at the highest concentrations tested (4000 and 5000 μg/ml). In the presence of metabolic activation, the drug was weakly mutagenic at concentrations of 1000 μg/ml and higher. In an *in vitro* cytogenetic study performed in cultured human lymphocytes, zidovudine induced dose-related structural chromosomal abnormalities at concentrations of 3 μg/ml and higher. No such effects were noted at the two lowest concentrations tested, 0.3 and 1 μg/ml.

Pregnancy: Pregnancy Category C. An oral teratology study in pregnant rats using doses up to 20 times the human dose has revealed no evidence of harm to the fetus due to zidovudine. It is not known whether zidovudine can cause fetal harm when administered to a pregnant woman or can affect reproductive capacity. Zidovudine should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether zidovudine is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions from zidovudine in nursing infants, mothers should be instructed to discontinue nursing if they are receiving zidovudine.

Pediatric Use: Safety and effectiveness in children have not been established.

ADVERSE REACTIONS: The most frequent adverse events and abnormal laboratory values reported in the placebo-controlled clinical trial of oral zidovudine administration in 281 patients (144 patients zidovudine; 137 patients placebo) were granulocytopenia and anemia. The occurrence of these hematologic toxicities was inversely related to CD4 (T4) lymphocyte number, hemoglobin, and granulocyte count at study entry, and directly releated to dose and duration of therapy. The frequency of granulocytopenia and anemia according to the patients' CD4 (T4) levels is shown in the following table:

	Pretreatment CD4 (T4) Levels			
	$\leq 200/mm^3$		$>200/mm^3$	
Abnormality	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia ($< 750/mm^3$)	47%	10%	10%	3%
Anemia (Hgb <7.5 g/dl)	30%	6%	3%	0%

Because many patients were anemic and/or granulocytopenic before starting therapy with zidovudine, examining the degree of change when compared to baseline, as shown in the table below, may be more informative.

		Pretreatment CD4 (T4) Levels			
		$\leq 200/mm^3$		$>200/mm^3$	
Abnormality	% Decrease from Baseline	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia	>50%	55%	19%	40%	13%
Anemia	>25%	45%	14%	10%	10%

The anemia appeared to be the result of impaired erythrocyte maturation as evidenced by increasing macrocytosis (MCV) while on drug.

The 281 patients treated in this controlled trial had serious underlying disease with multiple baseline symptoms and clinical abnormalities. The following table summarizes those reported clinical adverse events which occurred in at least 5% of all patients treated with zidovudine. Severe headache, nausea, insomnia and myalgia were reported at a significantly greater rate in zidovudine recipients.

Percentage (%) of Patients with Clinical Events					
Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %	Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %
BODY AS A WHOLE			MUSCULOSKELETAL		
Asthenia	19	18	Myalgia	8	2
Diaphoresis	5	4	NERVOUS		
Fever	16	12	Dizziness	6	4
Headache	42	37	Insomnia	5	1
Malaise	8	7	Paresthesia	6	3
GASTROINTESTINAL			Somnolence	8	9
Anorexia	11	8	RESPIRATORY		
Diarrhea	12	18	Dyspnea	5	3
Dyspepsia	5	4	SKIN		
GI Pain	20	19	Rash	17	15
Nausea	46	18	SPECIAL SENSES		
Vomiting	6	3	Taste Perversion	5	8

Clinical adverse events which occurred in less than 5% of all patients treated with zidovudine are listed below. Since many of these adverse events were seen in placebo-treated patients as well as zidovudine recipients, they may not be attributable to the drug.

Body as a whole: body odor, chills, edema of the lip, flu syndrome, hyperalgesia, back pain, chest pain, lymphadenopathy.
Cardiovascular: vasodilation.
Gastrointestinal: constipation, dysphagia, edema of the tongue, eructation, flatulence, bleeding gums, rectal hemorrhage, mouth ulcer.
Musculoskeletal: arthralgia, muscle spasm, tremor, twitch.
Nervous: anxiety, confusion, depression, emotional liability, nervousness, syncope, loss of mental acuity, vertigo.
Respiratory: cough, epistaxis, pharyngitis, rhinitis, sinusitis, hoarseness.
Skin: acne, pruritus, urticaria.
Special senses: amblyopia, hearing loss, photophobia.
Urogenital: dysuria, polyuria, urinary frequency, urinary hesitancy.

DOSAGE AND ADMINISTRATION: The recommended starting dose of Retrovir (zidovudine) is 200 mg (two 100 mg capsules) administered orally every four hours around the clock.

Hematologic toxicities appear to be related to pretreatment bone marrow reserve and to dose and duration of therapy. Careful monitoring of hematologic indices every two weeks is recommended to detect serious anemia or granulocytopenia. In patients with hematologic toxicity, reduction in hemoglobin may occur as early as 2 to 4 weeks, and granulocytopenia usually occurs after 6 to 8 weeks.

Dose Adjustment: Significant anemia (hemoglobin of <7.5 g/dL or reduction of >25% of baseline) and/or significant granulocytopenia (granulocyte count of $<750/mm^3$ or reduction of >50% from baseline) may require a dose interruption until some evidence of marrow recovery is observed. For less severe anemia or granulocytopenia, a reduction in daily dose may be adequate. In patients who develop significant anemia, dose modification does not necessarily eliminate the need for transfusion. If marrow recovery occurs following dose modification, gradual increases in dose may be appropriate depending on hematologic indices and patient tolerance.

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Essential Coverage Helps You Effectively Manage Hematologic Problems

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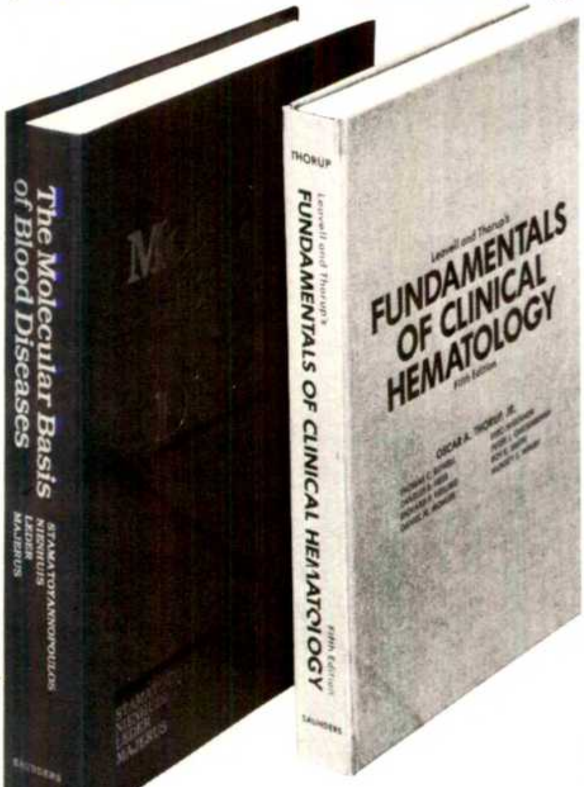

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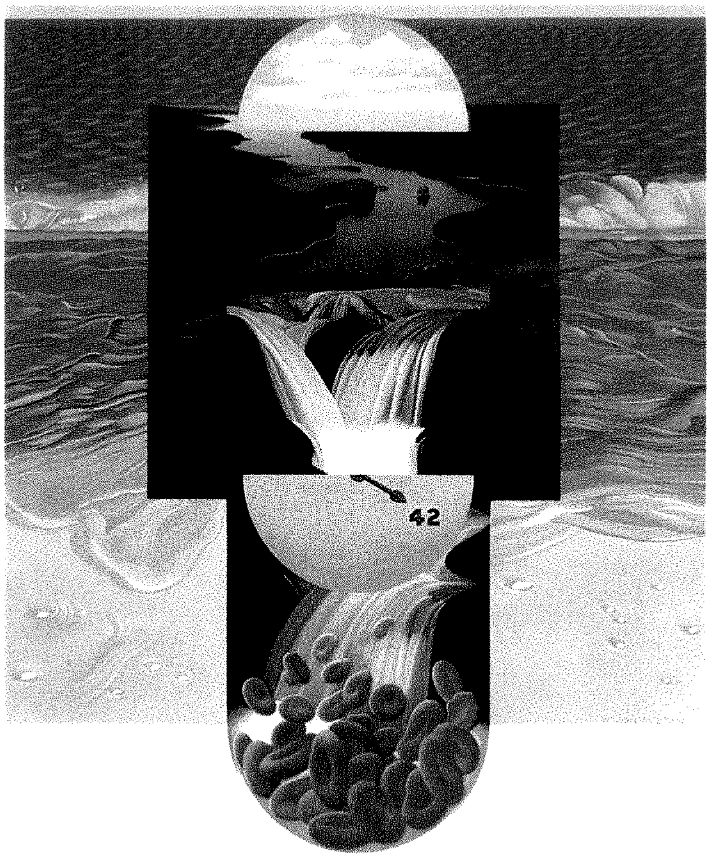

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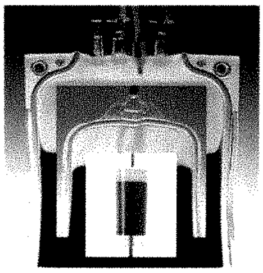

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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

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2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. Semin Psychiatry (in press)

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3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

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4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

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6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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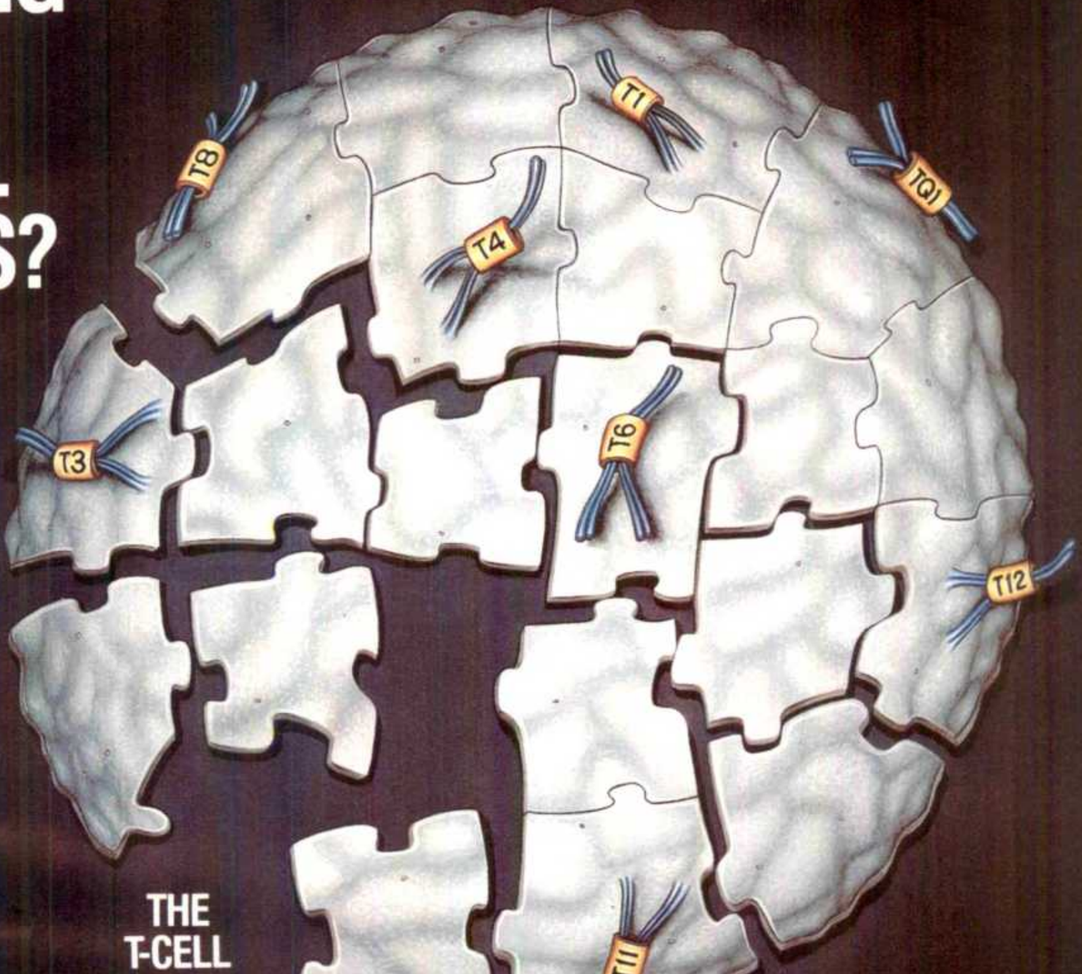

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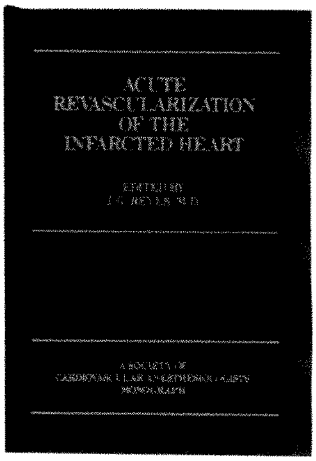

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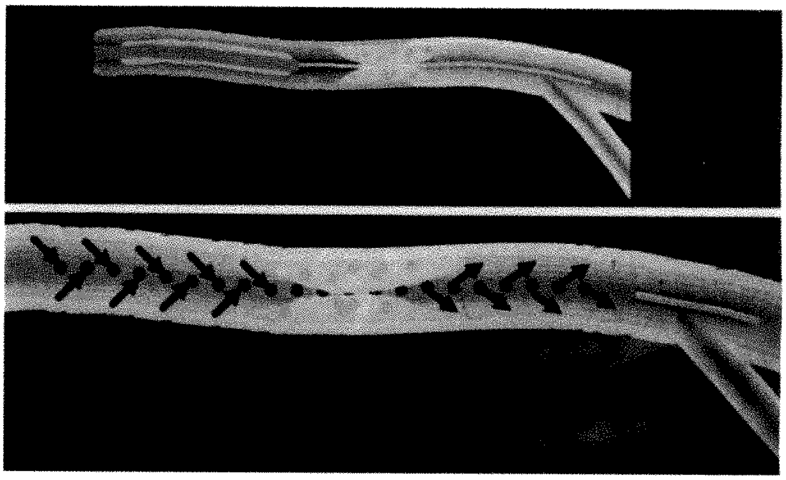

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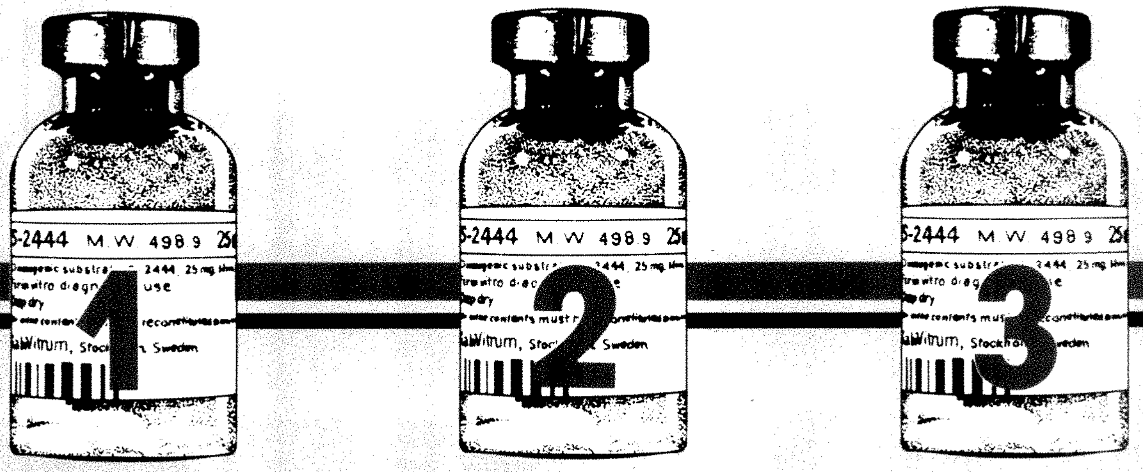

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Radiogenic mucositis or herpes simplex?

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INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) sterile powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: **1.** C.D. Mitchell, *et al.*, Lancet *1*(8235): 1389-1392, Jun. 27, 1981. **2.** Wade, *et al.*, Ann. Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am J Med. *73*(1A); 229-235, Jul. 20, 1982. **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *1*(8274): 697-700, Mar. 27, 1982. **6.** Z.M. Naib, *et al.*, Cancer Res. *33*:1452-1463, 1973.

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NEW JOURNAL FOR 1987

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WARNING

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Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the post-operative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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Heparin Binding Defect in a New Antithrombin III Variant: Rouen, 47 Arg to His

By M.C. Owen, J.Y. Borg, C. Soria, J. Soria, J. Caen, and R.W. Carrell

Antithrombin III (AT-III) Rouen is a hereditary abnormal antithrombin with normal progressive inhibitory activity and reduced heparin cofactor activity. It was isolated from the plasma of a woman who suffered a sudden idiopathic sensorineural hearing loss and balance impairment. There was no familial history of thrombosis. By heparin-Sepharose chromatography, AT-III Rouen was separated from the normal antithrombin on elution with increasing concentrations of NaCl. AT-III Rouen eluted earlier than is normal at both pH 7.4 and pH 6.0. At the lower pH, the antithrombins bound more avidly to the column, with the abnormal AT-III eluting closer to the normal than at the higher pH. Two-dimensional peptide mapping of tryptic and *Staphylococcus aureus* V8 protease digests of carboxymethylated antithrombins was performed on thin-layer silica plates. The abnormal peptide was located by tryptophan staining, and amino acid analysis and sequence studies demonstrated a substitution of an arginine at residue 47 for a histidine. Results from this study suggest that replacement of arginine 47 by a partially positively charged histidine has less effect on the heparin binding affinity than does replacing it with a neutral cysteine side chain as in AT-III Toyama, in which no heparin binding was observed. In addition, heparin binding per se is not a sufficient condition to activate AT-III.

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ANTITHROMBIN III (AT-III) is a member of the serpin family of plasma protease inhibitors and is the major inhibitor of thrombin.[1] It is activated by heparin to increase dramatically its association rate with thrombin.[2] Reduced plasma AT-III activity predisposes to thromboembolic disorders. Conversely, as was seen with a unique mutation in a homologous protein, antitrypsin Pittsburgh, a high plasma antithrombin activity results in prolonged clotting times.[3] Although many variants of AT-III have been reported, to date only three have been fully characterized. They are one of two types, corresponding to two major functional sites of AT-III. The first and more common type involves amino acid substitutions in the heparin binding area such as are found with the variants, Toyama (47, Arg to Cys)[4] and Basel (41, Pro to Leu).[5] These mutations result in a decreased activation by heparin but do not affect heparin-independent or progressive thrombin inhibition. The second type are those that have substitutions in the reactive center loop as occurs in AT-III Denver (394, Ser to Leu).[6]

A heparin binding site involved in the mediation of the heparin activation of AT-III appears to be located at residues 41, 47, and 49.[4,5,7] The nature and extent of the heparin binding site and the mechanism of activation of the molecule by heparin are not known. Neither is it known whether binding and activation are necessarily linked, ie, whether binding can occur without activation or vice versa.

We describe in this article the characterization of a new AT-III variant, AT-III Rouen, in which the arginine at residue 47 is replaced by a histidine. This variant binds heparin less avidly than is normal and is incompletely activated by it, but has normal heparin-independent activity.

MATERIALS AND METHODS

Antithrombin-III assays. The plasma concentration of immunoreactive antithrombin was determined by the method of Laurell.[8] Functional studies were performed, with and without heparin, using synthetic chromogenic substrates of thrombin and factor Xa, according to the method described by Ødegard and colleagues[9] and Abildgaard and co-workers.[10] In the actual procedure the reagent kit of Diagnostica Stago was used (Asnières, France) with 0.3 mol/L of Tris pH 8.7 buffer containing 3 IU/mL of heparin.

Heparin binding and activation of AT-III. Studies on the relationship between heparin binding to and activation of normal and abnormal AT-III were performed with varying amounts of heparin using Chromozym TH (Boehringer, Mannheim, FRG) substrate in phosphate-buffered NaCl buffers at pH 7.4 at isotonic, two times isotonic, and four times isotonic ionic strengths. Typically,

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to 600 μL of buffer containing 0.1 U, 5.0 U, or 50 U of heparin (mucous, Evans Medical, Liverpool, UK) and 0.5 μL of plasma 5 μL of thrombin was added (Parke Davis, Detroit, MI; topical bovine, diluted 15 μL to 1 mL). This was allowed to react for 20 minutes; 25 μL of substrate 5 mg/mL was then added, and the increase in absorbance at 405 nm was monitored.

Purification of AT-III. AT-III was isolated on heparin-Sepharose as described by McKay.[11] Typically, to 100 mL of plasma was added 1 mL each of 5 mol/L of $CaCl_2$ solution and 10% dextran sulphate (mol wt 500,000) solution. The supernatant was loaded onto a heparin-Sepharose column (1.5 × 30 cm), which was equilibrated with a buffer of 0.05 mol/L of Tris, 0.01 mol/L of citrate, and 0.15 mol/L of NaCl pH 7.4. The antithrombins were eluted with a linear NaCl gradient to 1.5 mol/L. Further purification was accomplished by ion-exchange chromatography on diethylaminoethyl (DEAE)-Sephadex A50 in 0.1 mol/L of Tris, 0.1 mol/L of NaCl pH 8.6, using a linear NaCl gradient to 0.45 mol/L.

Heparin-antithrombin binding at pH 6.0. The comparative binding to heparin-Sepharose of normal AT-III and Rouen at pH 7.4 and at pH 6.0 was investigated. The conditions above were used for the pH 7.4 experiment; however, at pH 6.0, a buffer consisting of 0.01 mol/L of sodium citrate and 0.01 mol/L of EDTA pH 6.0 was used.

Electrophoresis on agarose and sodium dodecyl sulfate polyacrylamide gels. Electrophoresis was carried out on 1% agarose gel at pH 8.6 in 0.075 mol/L of barbital buffer and at pH 6.0 in 0.01 mol/L of sodium citrate and 0.01 mol/L EDTA as described by Johansson.[12] Molecular weights were determined by electrophoretic mobilities in 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gels according to the method of Laemmli.[13]

^{14}C-Carboxymethylation and proteolytic digestion. Typically, 5 mg of AT-III was dissolved in 1 mL of 0.3 mol/L of Tris pH 8.0 in 8 mol/L of urea. Ten microliters of dithiothreitol (10 mg/mL) sufficient to give a 2 molar excess over the thiol groups of AT-III was added, and the solution was flushed with nitrogen and left at room temperature for 5 hours. Five microcuries of ^{14}C-iodoacetic acid (Amersham, Buckinghamshire, UK) was added, followed 30 minutes later by a three fold excess of nonradiolabeled iodoacetic acid (0.4 mg). After a further 30 minutes, the labeled protein was dialyzed against distilled water.

Tryptic digestion was performed at pH 8.6 in 0.1 mol/L of ammonium bicarbonate with trypsin at 37°C for 2 hours. Digestion with *Staphylococcus aureus* V8 protease was made at pH 7.8 in 0.1 mol/L of ammonium bicarbonate for 18 hours at 37°C.

Peptide mapping. Essentially, the method of Powers and colleagues[14] was followed. Baker-flex (J.T. Baker Chemical, Phillipsburgh, NJ) silica gel 1B plastic backed 20 × 20 cm plates with a layer thickness of 200 μm were prewashed by being chromatographed overnight in pH 6.4 electrophoresis buffer, pyridine:acetic acid:water (200:8:1,800). After being dried and reactivated at 110°C for 30 minutes, 3 to 5 nmol of enzyme digest was loaded, and the plate was chromatographed overnight in *n*-propanol:ammonium hydroxide (7:3). The plate, after drying, was electrophoresed at pH 6.4 at 500 V for 50 to 90 minutes.

Peptides were located by being stained with fluorescamine (Hoffmann-La Roche, Basel) or cadmium-ninhydrin, and the Ehrlich stain was used for tryptophan-containing peptides.[15] ^{14}Carboxymethyl-cysteine peptides were identified by autoradiography for 4 to 8 days at −80°C.

Amino acid analysis was done on peptides located by fluorescamine.[16] A small area (1 × 3 mm) of the silica from the central region of the peptide was scraped off, and the peptide was extracted in 2 × 25 μL of pH 6.4 electrophoresis buffer and dried. After hydrolysis, the amino acids were derivatized with phenylisothiocyanate and analyzed according to the method of Bidlingmeyer[17] by reverse-phase high-performance liquid chromatography (HPLC).

HPLC peptide separations. Peptides were separated on a C-18 column (Waters radial compression module) in 49 mmol/L of phosphate pH 2.9 and eluted with an increasing percentage of acetonitrile. Peptides were collected, dried over nitrogen, and further purified and desalted by rechromatographing using 0.1% trifluoroacetic acid, eluting with an increasing acetonitrile concentration. Peptides thus isolated were used for sequence studies.

Peptide sequencing. Peptides were sequenced using the gas phase sequencing method on a model 470A protein sequencer from Applied Biosystems; the PTH-amino acids were identified by HPLC.[18]

Case report. A 40-year-old woman was admitted to the hospital because of sudden idiopathic sensorineural hearing loss and balance impairment. Laboratory studies showed that the plasma concentration of immunoreactive AT-III was normal (111% of normal pool), but the AT-III activity in the presence of heparin was reduced to 55% of normal as measured with the chromogenic substrate CBS 3447. Although there was no familial history of thrombosis, one of her two daughters, aged 14 years, also showed reduced AT-III activity.

RESULTS

In both the propositus and her daughter, the heparin cofactor activities were significantly reduced, being 55% and 62%, respectively, of a normal pool. The progressive antithrombin and anti-factor Xa activities were normal, as were the plasma antigenic levels of AT-III determined by electroimmunoassay (Table 1).

The results of heparin-Sepharose chromatography of the patient's plasma at pH 7.4 and pH 6.0 (Fig 1) show heterozygosity for an antithrombin of decreased heparin binding affinity. Normal AT-III eluted, at pH 7.4, with 0.8 mol/L of NaCl, and the abnormal protein eluted with reduced heparin affinity with 0.45 mol/L of NaCl. At pH 6.0, the normal AT-III eluted with 1.1 mol/L of NaCl and the abnormal protein eluted with 0.9 mol/L of NaCl. Hence, the difference in NaCl eluting concentrations between the two AT-IIIs at pH 7.4 (0.35) is significantly higher than at pH 6.0 (0.2).

The thrombin inhibitory activity at pH 7.4 of normal plasma and plasma from the propositus with varying amounts of heparin and ionic strength buffers is shown in Fig 2. Under all conditions, the inhibitory activity of normal AT-III exceeded AT-III Rouen. AT-III Rouen activity was preferentially impaired by low levels of heparin and high ionic strengths as compared with normal AT-III.

The mol wt of both proteins was identical at 56,000.

Table 1. Antithrombin-III Assays of Rouen Plasma Expressed as a Percentage of Normal Plasma

	Anti-IIa Activity		Anti-Xa Activity		Antigenic Level
	Hep Cof	Progr	Hep Cof	Progr	
Chromogenic substrate	CBS 3447	S2238	S2222	CBS 3139	
Propositus	55	—	—	120	111
Daughter	62	110	75	130	115

Hep Cof, heparin cofactor; Progr, progression.

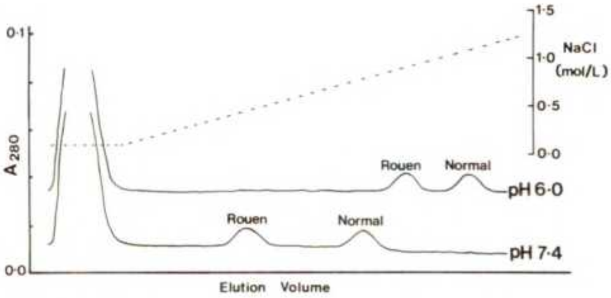


Fig 1. Elution profile of heparin-Sepharose chromatography of patient's plasma (heterozygous) showing the resolution of AT-III Rouen from normal antithrombin-III (AT-III) at pH 6.0 and pH 7.4. The proteins were eluted with an increasing linear NaCl gradient.

Electrophoresis on agarose gel showed the abnormal AT-III to migrate more anodally than the normal AT-III at pH 8.6, however, but to migrate essentially with the normal protein at pH 6.0 (Fig 3).

Numbers of peptide maps were prepared, and peptide analyses were performed to show only the one consistent difference, in peptides containing tryptophan 49. Maps of tryptic peptides revealed three tryptophan-containing peptides in the normal map but only two in the abnormal map. Amino acid analyses identified normal tryptic peptide 29 (residues 223 through 226) in both maps, but the identity and composition of the other two tryptophan peptides could not be conclusively determined because of contamination by neighboring or superimposed peptides. The V8 maps, however, gave two well-separated tryptophan peptides from both normal and abnormal proteins. A clear chromatographic shift and a minor electrophoretic shift had occurred in one of the peptides from the abnormal map. Amino acid analysis identified the peptide as residues 43 through 50 with, in the abnormal map, a histidine replacing one of the two arginines (Table 2). The sequence analysis of this peptide isolated by HPLC revealed that the arginine at residue 5 in the peptide was replaced by a histidine. This corresponds to position 47 in the intact protein.

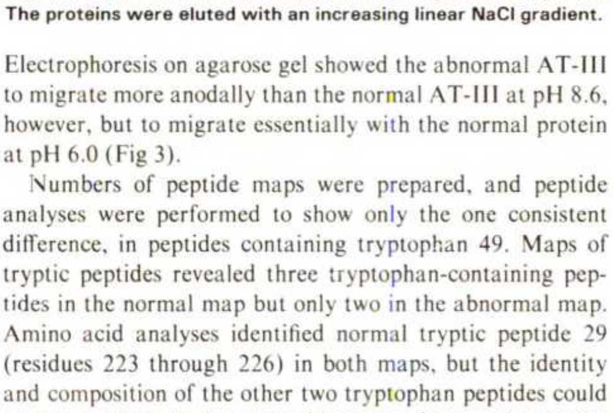


Fig 2. Antithrombin activity of control plasma containing only normal antithrombin-III (AT-III) (normal AT-III) and patient's plasma heterozygous for normal and Rouen AT-III in isotonic, two times isotonic and four times isotonic (the inhibitory activity of patient's plasma was below measurable limits) buffers in the presence of 0.1, 5.0, and 50 U of heparin. Results are expressed as a percentage of normal AT-III activity, which is caculated as the average activity for the three heparin concentrations of normal AT-III in isotonic buffer.

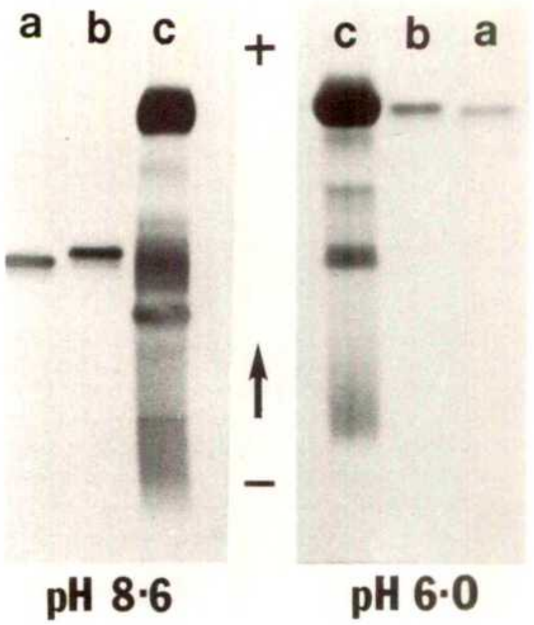


Fig 3. Electrophoresis in agarose gel at pH 8.6 and pH 6.0 of purified normal antithrombin-III (AT-III) (a), purified Rouen AT-III (b), and normal human serum (c).

DISCUSSION

We report here a new variant of AT-III, AT-III Rouen, that has decreased heparin binding affinity. The replacement of the normal arginine at position 47 by a histidine modifies the binding avidity for heparin at normal physiological pH. Whereas normal AT-III eluted with 0.8 mol/L of NaCl at pH 7.4, AT-III Rouen eluted at 0.45 mol/L of NaCl, a difference of 0.35 mol/L. At this pH, the histidine side chain would not be expected to be positively charged. This positive charge is likely to be restored to that which normally occurs with the arginine at position 47 when the pH is lowered to 6.0, which is below the usual pK_a of free surface histidine side chains in proteins.[19] This is demonstrated (Fig 3) by the electrophoresis at pH 6.0 at which the mobility of AT-III Rouen was essentially restored to that of the normal protein.

Table 2. Amino Acid Analysis of Tryptophan-Positive V8 Peptides (Arrows, Fig 5)

Peptide	Normal	Rouen
Asp	0.7	1.0
Glu	1.0	1.3
His	0.0	0.8
Arg	1.9	0.9
Thr	1.0	0.9
Ala	1.1	1.1
Val	0.8	0.7

Yield/residue = 65 pmol.

The difference in binding affinities between the normal and Rouen at this pH is indicated by the decrease in elution concentration to 0.2 mol/L of NaCl with the normal eluting at 1.1 mol/L and Rouen eluting at 0.9 mol/L of NaCl. Both antithrombins bind more tightly to heparin at pH 6.0 as compared with pH 7.4. Indeed, AT-III Rouen at pH 6.0 binds even more avidly than the normal at pH 7.4. Clearly, the reduction in the pH increased the overall protonation of the antithrombins, resulting in a stronger ionic attraction to the negatively charged heparin. AT-III Rouen, having an extra protonizable residue moves closer to the normal heparin affinity after the drop from pH 7.4 to 6.0.

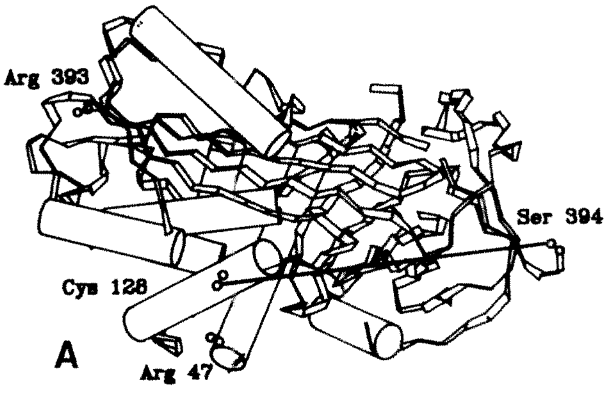
Yet, at pH 6.0, there still remains a difference in binding affinity between the protonated histidine 47 at AT-III Rouen and the normal AT-III. Because AT-III Rouen has an electrophoretic mobility essentially indistinguishible from normal AT-III at pH 6.0, incomplete protonation of the histidine seems unlikely. The results suggest that steric considerations are important and that heparin binding is not simply a charge effect. AT-III Toyama[4] is a mutant involving this same residue, 47, but has arginine replaced by a cysteine. Like AT-III Rouen, it has normal progressive activity but, although data were not given, Koide reported that AT-III Toyama has a total loss of heparin binding activity. This is surprising since in AT-III Rouen heparin binding is not solely dependent on this one residue. The new cysteine in AT-III Toyama forms a disulphide bond with free plasma cysteine,[4] and this may sterically block heparin binding.

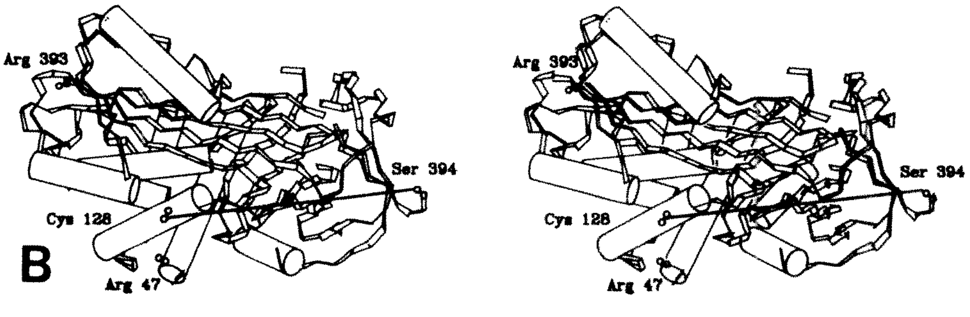
If the heparin binding of AT-III to the Sepharose-linked heparin reflects the binding in vivo, it seems clear that under physiological conditions AT-III Rouen still binds heparin, albeit less avidly than normal, but puzzlingly is not completely activated by the heparin. This raises the question of whether heparin binding per se is a sufficient condition to activate the molecule. The experiment assessing the activities of normal AT-III and AT-III Rouen at pH 7.4 in varying ionic strength buffers, with differing amounts of heparin, showed (Fig 2) that under normal isotonic conditions the plasma heterozygous for Rouen antithrombin averaged 73% of the activity of normal, whereas in twice isotonic buffer this dropped to 59%. At four times isotonic strength, AT-III Rouen was not measurably activated by heparin even at high concentrations. By comparison, normal AT-III was activated by the intermediate and high heparin levels but only to 12% and 24%, respectively, of the levels seen in normal isotonic conditions. The effect on activity of varying the heparin concentration was seen most dramatically in the two-times isotonic buffer in which the activity of AT-III Rouen increased 42% from low to intermediate heparin levels.

Hence, there is a competition between the charged heparin molecule and the buffer ions for the charged binding site regions on the AT-III. The normal AT-III with a greater heparin binding affinity can be fully activated with lower heparin levels than AT-III Rouen. Yet with high heparin levels and isotonic conditions, AT-III Rouen is not completely activated by the heparin binding. A distortion of the heparin binding site, although still enabling binding, may produce an effect distal to this site, resulting in misalignment of the heparin at some distant site and a consequent inability to activate the AT-III.

The mechanism of heparin activation of AT-III is still unknown. Recent homology studies[20] of the family of inhibitors to which AT-III belongs, the serpins, indicate that AT-III has substantially the same secondary structure as α_1-antitrypsin and hence has a similar tertiary structure. The crystallographic structure of post complex, cleaved, α_1-antitrypsin as determined by Loebermann and colleagues[21] is

Fig 4. Theoretical projection of antithrombin-III (AT-III) based on known structure of post-complex α_1-antitrypsin [(A) mono; (B) stereo]. Antithrombin has a flexible amino terminal tail of 45 residues hinged near the heparin binding site Arg 47. Heparin has been suggested to interact with this tail to reveal the active center (deduced[20,21] to be situated near Ser 394 in the intact molecule). The molecular model shows this to be unlikely because only seven residues of the tail are free to move from the cystine disulphide bond 8-128 allowing bridging of 27.2 Å v the minimum 35.38 Å (line from 128 to 394).





shown in Fig 4, with the structure of AT-III superimposable on it with only minor modifications. There must, however, be a significant structural difference between the two to explain the masking of inhibitory activity that occurs in AT-III but not in α_1-antitrypsin. The prime candidate is the N terminal tail of AT-III, which is longer than that of α_1-antitrypsin and has minimal homology with it. Furthermore, the mutation in antithrombin Rouen at Arg 47 is just past the base of the tail and the beginning of the first (A) helix. The longer N terminal tail of AT-III is held in its position on the external surface of the molecule by two disulphide bridges. The first one bridges residue 8 of the tail to residue 128, which is residue 14 of the D helix. The second bridges residue 21 of the tail to residue 95, which is sandwiched between the last residue of helix B and the first residue of helix C. This means that the N terminal tail must lie on the opposite side of the molecule to the deduced position of the reactive loop; thus, it is unlikely to shield directly or cover the reactive center of the molecule.

Further studies of this mutant and molecular characterization of variant antithrombins, particularly those of the class that have normal progressive activity but no heparin cofactor activity, should shed more light on the mechanism of heparin binding and activation of AT-III.

ACKNOWLEDGMENT

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Clinical Investigation of Human Alpha Interferon in Chronic Myelogenous Leukemia

By Moshe Talpaz, Hagop M. Kantarjian, Kenneth B. McCredie, Michael J. Keating, Jose Trujillo, and Jordan Gutterman

Fifty-one patients with previously untreated or minimally treated chronic myelogenous leukemia in chronic phase received human alpha interferon 3 to 9 × 10^6 units intramuscularly (IM) daily until complete hematologic remission, then at doses ranging from 3 × 10^6 units every other day to 9 × 10^6 units daily. Forty-one (80%) patients achieved a hematologic response, 36 (71%) of them attaining a complete hematologic remission with normal peripheral WBC and differential counts. Responding patients showed continuous but slow normalization of several other blood and marrow parameters including platelet counts, serum lactic dehydrogenase and B12 levels, and marrow cellularity and maturation index. Suppression of the Philadelphia chromosome on serial cytogenetic studies of marrow metaphases was documented in 20 of the 36 patients who achieved complete hematologic remission (56%; 39% of total group), eight of whom (22%) had a decrease of the Philadelphia chromosome-positive metaphases to less than 35%. These changes were persistent for 6 months or longer in 18 patients, seven of whom had continuous suppression of the Philadelphia chromosome to less than 90% for a median of 30+ months (range 21+ to 39+ months). After a median follow-up period of 37 months, 25 patients remain in continued disease control with interferon therapy. The projected 3-year survival rate is 76%, with a yearly death rate of 6%, 9%, and 9% in the first 3 years. Response, Philadelphia chromosome suppression, and survival were significantly better among patients in the low-risk category compared to intermediate- and high-risk categories, as defined by a multivariate analysis-derived prognostic model. The projected 3-year survival rate was 94% for patients who achieved a complete hematologic remission on interferon therapy and 45% for those who did not. Thirteen patients have developed blastic crisis, six with lymphoid and three with undifferentiated morphology. We conclude that human leukocyte alpha interferon effectively controls chronic myeloid leukemia and allows reappearance of diploid hemopoietic cells in some patients.

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CHRONIC myelogenous leukemia (CML) disease has a biphasic clinical course. The initial chronic phase is easily controlled with therapy and lasts for a median of 2 to 4 years. This then evolves into a phase of resistant, rapidly fatal blastic crisis.[1,2] Various therapies have resulted in significant but limited progress. The median survival after diagnosis has improved from 19 months for untreated patients[3] to a range of 30 to 45 months for those receiving single agents such as myleran or hydroxyurea[4-7] and 45 to 55 months for patients treated with intensive combination chemotherapy.[8-10] However, the progressive and ultimately fatal nature of CML has not been altered significantly; most patients still die from blastic crisis, with no consistent cure fractions recorded.[1,2,11] Allogeneic bone marrow transplantation is a promising approach in CML because of its curative potential, but its application and optimal timing are limited by donor availability, patient age, and the high incidence of morbidity and mortality in a disease that has a long chronic phase.[12] These factors emphasize the need for new modalities to treat patients with CML.

Interferons have opened new possibilities for managing patients with cancer.[13,14] Human alpha interferon (IFN-α), produced by virus-stimulated leukocytes, has undergone extensive trials and has shown promising results in several tumor categories including hairy cell leukemia and renal cell cancer.[15-17]

We have previously reported on our preliminary studies with IFN-α in CML showing its cytoreductive activity.[18] The activity of interferon in CML has also been documented recently using the pure recombinant alpha interferon material.[19] In this report we summarize our long-term results with the partially pure IFN-α in patients with Philadelphia chromosome-positive chronic-phase CML, evaluating efficacy and toxicity, describing the cytogenetic changes associated with therapy, and analyzing patterns of blast crisis, mortality rates, and overall prognosis.

PATIENTS AND METHODS

Population study. Between August 1981 and December 1984, 51 patients with Philadelphia chromosome-positive chronic-phase CML entered the study after giving informed consent. All patients were advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent. Eligibility criteria required the presence of less than 30% blasts in peripheral blood and bone marrow, absence of extramedullary disease, and minimal previous therapy. The patients' characteristics are detailed in Table 1. The median age was 42 years (range 20 to 70 years). The majority of patients had received no therapy, and 20 had been treated with single-agent chemotherapy for periods of less than a month. Forty-six patients (90%) started therapy within six months after the diagnosis. The patients' classification into low-, intermediate- and high-risk categories, based on a multivariate analysis model of pretreatment characteristics, was similar to that of the general CML population.[10] Forty-eight patients had the common 9;22 balanced translocation. Among these, two had evidence of disease acceleration with cytogenetic clonal evolution: one had additional chromosomal

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Table 1. Patient Population

Characteristics	No. of Patients	(Percentage)
Total number	51	(100)
Male sex	34	(67)
Age 60 years or older	8	(16)
Splenomegaly	36	(71)
Hemoglobin < 12g/dL	29	(57)
Platelet count > 700 × $10^3/\mu L$	13	(25)
Peripheral WBC count > 50 × $10^3/\mu L$	32	(63)
Peripheral blasts and promyelocytes	34	(67)
Cytogenetic Pattern		
Translocation 9;22 only	46	(90)
Other translocations	3	(6)
Clonal evolution	2	(4)
Prognostic category		
Low risk	22	(43)
Intermediate risk	16	(31)
High risk	11	(22)
Not evaluable	2	(4)
Previous therapy		
None	31	(61)
Myleran or hydroxyurea	18	(35)
Others	2	(4)

material on the short arm of chromosome 16 (16p+) in 25% of the marrow metaphases; the other showed a balanced translocation between the long arms of chromosomes 8 and 17 [(t(8q−;17q+)] in 30% of marrow metaphases. The remaining three patients had Philadelphia-like translocations with the cytogenetic material being translocated between chromosomes 22 and 4 [t(4q+;22q−)], chromosomes 22 and 6 [t(6q+;22q−)], and chromosomes 22, 9, and 11 [t(9q;11p;22q)] in each, respectively.

Patient evaluation. Pretreatment evaluation included physical examination, complete blood counts (CBC), SMA 12, liver and renal function studies, determination of serum vitamin B12, lactic dehydrogenase (LDH) and leukocyte alkaline phosphatase (LAP) levels, bone marrow aspiration and biopsy, cytogenetic studies with Giemsa banding,[20] chest roentgenograms, and liver-spleen scan. Other studies were done as indicated by the patient's condition. Follow-up studies during the induction phase included physical examination and CBC two to three times weekly. After the cytoreductive results were observed, the frequency of studies was decreased to once every one to four weeks. A full workup, including bone marrow aspiration and biopsy, cytogenetic studies, vitamin B12 and LDH levels, LAP score and liver- spleen scan were done every 3 to 4 months during the first year and every 4 to 6 months thereafter. Serial cytogenetic studies were performed on at least 25 metaphases. If fewer than 25 metaphases were present in a particular specimen, a repeat bone marrow examination was done within one month. A marrow maturation index was defined as the ratio of the percentage of myelocytes, metamyelocytes, and granulocytes to the percentage of blasts and promyelocytes.

Treatment plan and dose modification. Induction therapy consisted of daily intramuscular injections of IFN-α (State Serum Institute, Helsinki, Finland) given at doses ranging from 3 × 10^6 units to 9 × 10^6 units daily until hematologic remission. For patients who achieved hematologic remission, the IFN-α dosage was reduced to levels varying from 3 × 10^6 units every other day to 9 × 10^6 units daily to maintain hematologic remission with a WBC count of less than 10,000/μL and a platelet count above 50,000/μL. Dosage modifications according to toxicity are detailed below.

Criteria for response, toxicity, and removal from study. Response criteria were defined as follows:

Complete hematologic remission: This required normalization of peripheral WBC counts to levels lower than 10,000/μL and normal differentials with no immature forms (blasts, promyelocytes, myelocytes, or metamyelocytes); normalization of platelet counts to less than 450 × $10^3/\mu L$; and disappearance of all clinical symptoms and signs of disease, including palpable splenomegaly. Maximal suppression of the Philadelphia chromosome was defined as four subgroups of (1) no cytogenetic response if the Philadelphia chromosome persisted in all analyzable metaphases, (2) minimal cytogenetic response if its expression was suppressed to percentages of 35% to 95%, (3) partial cytogenetic response if suppression was to levels of 5% to 34%, and (4) complete cytogenetic response if total elimination of the Philadelphia chromosome was noted in marrow metaphases.

Partial hematologic remission: This category was defined as a decrease in the WBC counts to at least 50% of pretreatment level and to at least below 20,000/μL; it included patients whose peripheral WBC counts had become normal but who had persistent splenomegaly or immature peripheral cells.

Failure: This group included all patients who had less than partial hematologic remissions.

Toxicity: Toxic reactions were graded as mild, moderate, severe, and life threatening on a scale of 1 to 4 (Table 2). The dose of interferon was reduced by 50% in patients with grade 2 toxicity. If toxicity persisted or was grade 3 or higher, interferon therapy was discontinued temporarily until the toxic effects reverted to grade less than 1. Grade 4 neurologic toxicity required permanent discontinuation of interferon.

Criteria for removal from the study. These included (1) development of blastic crisis with 30% or more blasts in the bone marrow or peripheral blood or the development of extramedullary disease; (2) failure to respond to therapy as evidenced by a continuous increase in WBC count or less than 25% decrease in WBC counts from pretreatment levels over a period of 14 weeks; (3) an initial response followed by subsequent increase in WBC count to more than 40,000/μL, uncontrolled by continued interferon therapy (patients with a cyclic peripheral leukocyte count pattern were continued on therapy unless the WBC counts were consistently higher than pretreatment values); and (4) unacceptable toxic reactions as defined above.

Statistical analysis. Survival and disease control duration curves were plotted by the method of Kaplan and Meier.[21] Disease control duration time was calculated from the date of start of therapy until development of resistance, toxicity, or blastic crisis. Tests of differences in survival distributions were based on a generalized Wilcoxon test.[22] Comparisons of differences among subgroups were made using chi-square tests for remission rates and degree of cytogenetic response.

RESULTS

Remission induction. All 51 patients in the study were evaluable with a median follow-up duration of 37 months. Induction therapy with IFN-α resulted in complete hematologic remission in 36 patients (71%). Five patients (10%) achieved a partial hematologic remission, three of these being classified as such because of persistent splenomegaly; and ten patients failed to improve (Table 3).

All 36 patients who obtained complete hematologic remission had, by definition, normalized peripheral WBC counts and differentials. The median time to achieve a complete hematologic remission was 14 weeks, with a range of 2 to 55

Table 2. Toxicity Criteria

Toxicity	1	2	3	4
Fever, chills, influenza-like symptoms; bone and muscle aches	Self-limiting, no therapy needed	Require antipyretics or anti-inflammatory agents	Require narcotics and/or hospitalization	Life threatening
Fatigue, performance status (Zubrod scale)	1	2	3	4
Weight loss (Percentage of body weight)	0-5	6-10	11-15	>15
Renal function (creatinine in mg/100 mL)		up to 2.5	>2.5	
Liver function				
Bilirubin (mg/100 mL)		up to 2.5	>2.5	
SGOT (mU/mL)		300-600	>600	
Neurologic	Does not interfere with daily activities or require medical intervention	Moderate impairment of activities, requires medical therapy	Severe impairment of activities, requires discontinuation of interferon and medical therapy	Coma, seizures, confusion, parkinsonism, severe depression
Hematologic				
Granulocyte count (per μL)		1,000-2,000	<1,000	
Platelet count ($\times 10^3/\mu$L)		50-100	25-50	<25

weeks. Two different patterns of response were observed. Twenty-six of the 36 patients (72%) had a steady gradual decline of peripheral counts and achieved remission after a median period of 9 weeks (range 2 to 28 weeks). The other ten patients (28%) manifested a cyclic pattern of response with WBC and platelet count in cycles of 4 to 8 weeks. Therapy induced lower peaks for WBC and platelet counts with each subsequent cycle; the median time to achieve remission in these patients was 26 weeks (range 8 to 55 weeks). Twenty-four of the 36 patients had had palpable splenomegaly that disappeared on follow-up studies. The responses of patients in each prognostic category were compared and are shown in Table 3.

Blood, marrow, and cytogenetic changes in patients who achieved complete hematologic remission. Changes in blood and marrow parameters in the first 2 years of therapy are shown for the 36 patients who achieved complete hematologic remission (Fig 1). Decline in WBC count was the earliest manifestation of response. Thrombocytosis with platelet counts of more than 500 × $10^3/\mu$L was present in 12 patients (33%), six of them having counts higher than 800 × $10^3/\mu$L. Platelet counts became normal in all responding patients. Subsequent increases in platelet as well as other parameters were noted among patients who failed after initial disease control, which accounts for the extreme values shown.

Serial bone marrow studies revealed a gradual decrease in bone marrow cellularity and an increase in maturation index. Marrow changes lagged behind peripheral blood changes: marrow cellularity declined from a median of 100% to a median of 75%, and the maturation index increased from a median of 8.3 before therapy to a median of 26.0 at 12 months (Fig 1).

Return of cells with diploid karyotype were noted with continued IFN-α therapy on serial cytogenetic studies of bone marrow cells. Twenty of the responding patients (56%, or 39% of the total group) manifested evidence of suppression of the Philadelphia chromosome on follow-up cytoge-

Table 3. Results of IFN-α Therapy

	No. of Patients in Risk Category* (Percentage)							
Response Category	Total		Low		Intermediate		High	
Complete hematologic remission	36(71)		18(82)		10(62)		7(64)	
Philadelphia status								
100%	16(31)		5(23)		3(19)		7(64)	
35%-95%	12(24)	39%	9(41)	59%	3(19)	44%	—	0%
5%-34%	3(6)		1(6)		2(13)		—	
0%	5(10)		3(14)		2(12)		—	
Partial hematologic remission	5(10)		2(10)		3(19)		—	
Resistant disease	10(20)		2(10)		3(19)		4(36)	
	51(100)		22(100)		16(100)		11(100)	

*Pretreatment characteristics were not available in two patients to determine their risk category: one had resistant disease, and one achieved complete hematologic remission but no suppression of the Philadelphia chromosome.

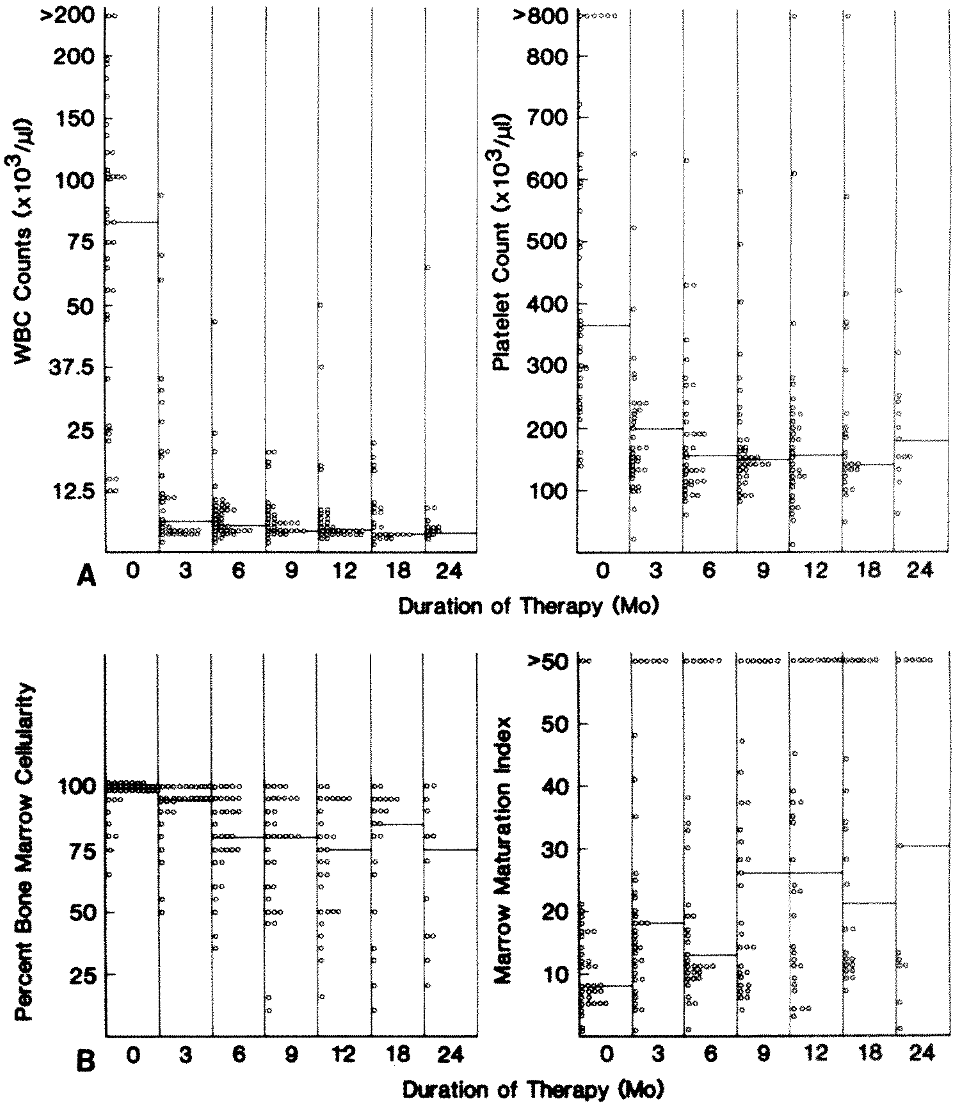

Fig 1. Changes in blood (A) and marrow (B) parameters with IFN-α therapy.

netic studies. Cytogenetic conversion to a partial diploid state was minimal (Philadelphia chromosome-positive in 35% to 95% of metaphases) in 12 patients (24%) and significant (Philadelphia chromosome-positive in less than 35% of metaphases) in eight patients (22% of responders, 16% of total). Suppression of the Philadelphia chromosome was slow, with changes observed after a median duration of 9 months of therapy (range 3 to 15 months). Cytogenetic conversion to a partial diploid state was short lived in two patients but persisted 6 months or longer in 18 patients, seven of whom showed evidence of continuous Philadelphia chromosome suppression for a median of 30+ months (range 21+ to 39+ months) (Table 4). The incidence of Philadelphia chromosome suppression was 59%, 44%, and 0% respectively, for low-, intermediate-, and high-risk groups ($P <$ 0.01) (Table 3).

Similar changes were documented for patients' serum LDH and vitamin B12 levels, which reverted slowly to normal values with response to therapy. Sixty-four percent of patients had had LDH values above 600 U/dL before therapy, compared to 12% after 3 months, 7% after 12 months, and 0% after 18 months of therapy. Similarly, although 94% of patients had had serum B12 levels above 1,000 pg/mL before treatment, this percentage decreased to 40% at 3, 25% at 12, and 14% at 18 months.

Disease control duration and failure patterns. The median duration of continued disease control with IFN-α was 29 months for the whole group (Fig 2), 33+ months for patients obtaining a complete hematologic remission, and 9 months for those achieving only a partial hematologic remission.

Patient outcome is summarized in Table 5. At the time of analysis, 25 of the initial 36 patients (78%) who obtained a complete hematologic remission remain in continued hematologic remission on IFN-α therapy, whereas 11 have failed: seven developed resistant disease and are all controlled on alternate therapies with hydroxyurea or myleran, and four evolved into blastic crisis. Of the 15 patients who achieved a partial remission or no response, ten were taken off the study while in benign phase (resistance 4, toxicity 1, both 5), four of them developed subsequent blastic crisis, two died in chronic phase, and four are alive on alternate treatments.

Table 4. Serial Cytogenetic Changes with IFN-α Therapy in 18 Patients with Persistent Philadelphia Chromosome Suppression for 6 or more Months

	Percentage of Philadelphia Chromosome-Positive Metaphases Months of Therapy										
Patient	3	6	9	12	15	18	21	24-27	30-33	36-39	42-45
1	100	90	95	100	90	80	70	50	10	5	0
2	70	IM*	IM	15	10	20	5	30	0		
3	100	70	70	55	80	—	0	0	5		
4	100	100	90	55	30	—	0	5	15		
5	100	100	IM	100	0	75	100				
6	100	75	80	80	—	10	5	5	10	100	
7	65	95	90	90	65	80	IM	95	25	100	30
8	100	—	100	75	55	20	90	80	100		
9	100	100	IM	IM	IM	95	—	35	95	95	
10	100	IM	82	70	IM	—	67	45	95		
11	100	100	—	65	—	55	—	86			
12	100	100	90	100	100	—	100	—	80	50	70
13	100	50	85	70	—	75	—	85	95	IM	
14	100	100	100	90	—	—	—	90	95		
15	100		100	100	90	45	95	90			
16	100	100	—	—	75	95	85	90			
17	100	IM	—	70	100	85	IM	85	100	100	100
18	100	80	75	90	100						

*IM = insufficient metaphases.

All but patient 18 are still on IFN-α therapy with continued disease control.

The other five patients who had partial remission or failed on therapy developed blastic crisis. Thus 11 of the 17 patients who showed resistance to interferon therapy in benign phase remain on alternative therapies with good disease control for a median of 20 months (range 12 to 35 months). Four developed subsequent blast crisis, and two other patients died, one of accelerated disease, the other of thoracocentesis-induced pneumothorax and empyema.

Thirteen patients have already developed blastic crisis after a median therapy duration of 11 months (range 2 to 30 months) (Fig 3). Blastic crisis has developed in 9 of the 15 patients who did not achieve a complete hematologic response compared to 4 of 36 who did (60% *v* 11%, $P < 0.05$). In the latter group three had no cytogenetic response, and one had a minimal cytogenetic response with a decrease of the Philadelphia-positive metaphases to 85% for 3 months only.

The patterns of blastic crisis and response to chemotherapy differed from previous experience. Six of the 13 patients had lymphoid blastic crisis, three had undifferentiated, and four had myeloid blast crisis morphologies. Five of the six patients who had lymphoid blast crisis achieved remission and entered a second chronic phase lasting for a median of three months (range 2 to 11+ months) with vincristine, doxorubicin (Adriamycin), and dexamethasone (VAD).

Toxicity. Early and late patterns of host toxic reactions were observed among patients treated with IFN-α (Table 6). Forty-seven of the 51 patients (92%) experienced early toxic reactions, most of which consisted of fever, chills, fatigue, anorexia, and muscle and bone pains. Severe early toxic reactions were unusual and did not require interruption of treatment in any patient. Other toxicities such as insomnia, excessive sweating, nervousness, postnasal drip, and mild depression were unusual. The toxic effects usually lasted for a median of three days (range one to 12 days), improved gradually with therapy, and were manageable with antipyretic and analgesic medications.

The majority of patients tolerated chronic therapy well, with 76% maintaining an average performance status of 0 (Zubrod scale) and continuing their normal activities. Twelve patients (24%) had mild chronic symptoms with an average performance status of 1 to 2. Overall, six patients (12%) had treatment discontinued because of late toxicities.

Serious late toxic reactions included myalgias; bone and joint pains; severe fatigue, weight loss, and decreased performance; neurotoxicity; and immune-mediated thrombocytopenia (Table 6). The myalgias that occurred at some point during therapy in 17 patients were generally diffuse. Patients usually responded to a decrease in interferon dosage and to analgesics. Bone pains occurred most frequently in the proximal upper and lower extremities, pelvis, and ribs. Temporary discontinuation of therapy and administration of analgesics and steroids allowed therapy to be resumed in eight of the 12 patients who had severe pains. Chronic severe fatigue, anorexia, weight loss, and poor performance were observed in four patients.

Neurotoxicity was manifested in several patterns, some of which have been reported.[23] Central nervous system (CNS) manifestations consisted of apathy or agitation, insomnia, withdrawal, bland affect, lack of interest, decreased attention span and short-term memory, and depression. These occurred mildly in nine patients, improving with methylphenidate in six, and severely in three patients, requiring treatment interruption. One of the two patients who showed parkinson-like signs and symptoms had concomitant severe depression, which led to discontinuation of therapy.

Among the four patients who developed immune-mediated thrombocytopenia with platelet counts lower than 50,000/

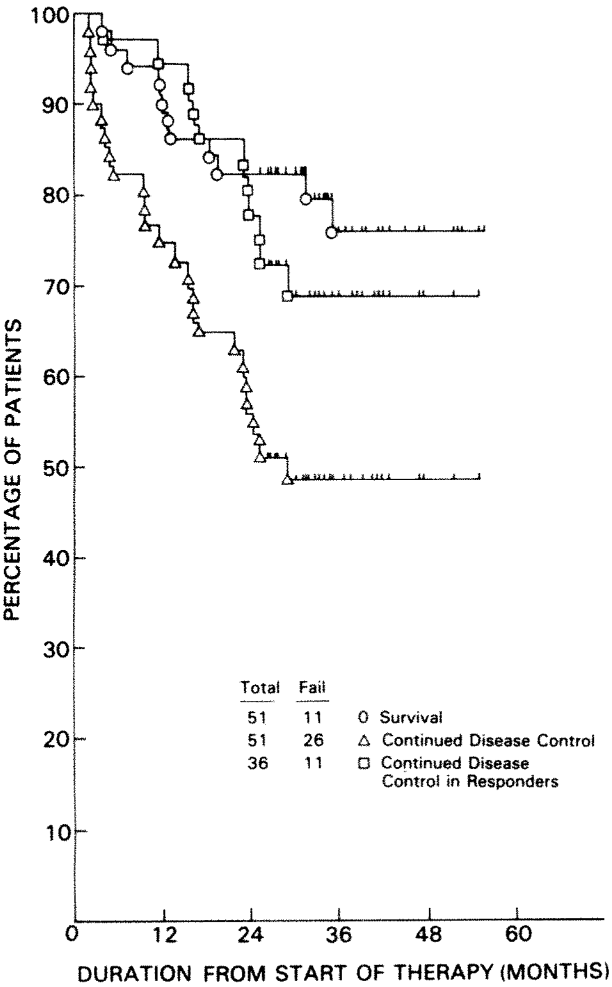

Fig 2. Duration of disease control and survival with IFN-α therapy.

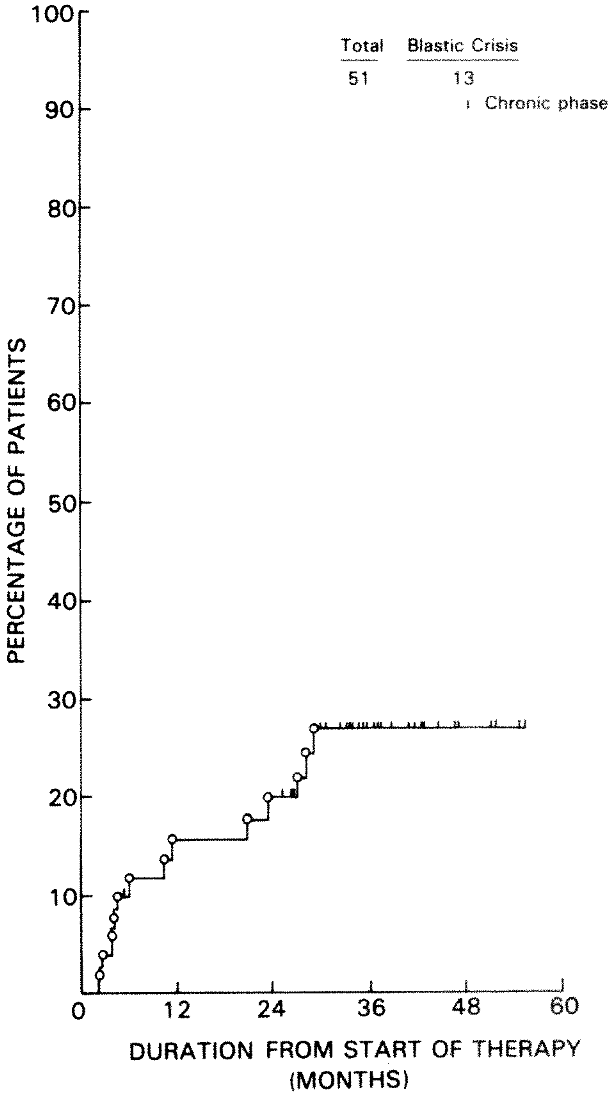

Fig 3. Cumulative incidence of blastic crisis in patients treated with IFN-α.

μL, platelet recovery was noted in two patients after discontinuation of interferon and institution of steroids; the other two patients underwent splenectomy for persistent thrombocytopenia with subsequent recovery of platelet counts. Other less common chronic toxic reactions included mild hair loss (18%), diarrhea (12%), headaches (14%), anorexia (10%), disturbed liver function (6%), postnasal drip (6%), cough, lung infiltrates, and impotence (2% each).

The incidence of severe toxic reactions increased with development of resistant disease. Five patients were taken off study because of resistant disease and increasing toxicity, which consisted of muscle or bone pains in four and neurotoxicity in one patient.

Table 5. IFN-α Therapy in CML: Present Status of Patients

	No. of Patients in Response Category (Dead)			
Status	Total	Complete Hematologic Remission	Partial Hematologic Remission	Resistant Disease
Continued disease control	25	25	0	0
Off therapy in benign phase	13 (2)	7 (0)	3 (0)	3 (2)
Blastic crisis	13 (9)	4 (2)	2 (2)	7* (5)
	51 (11)	36 (2)	5 (2)	10 (7)

*Includes four patients who discontinued therapy while in chronic phase and subsequently developed blastic crisis (see text).

Survival. After a median follow-up period of 37 months, 40 patients (78%) are alive, 36 being in chronic phase disease (25 of them on IFN-α therapy) and four in second chronic phase. The median overall survival has not been reached, and the projected three-year survival is 76% (Fig 2).

Relative yearly hazard rates for death were 6% during the first, 9% during the second, and 9% during the third year of study. The projected 3-year survival was 94% for patients with complete hematologic remission and 45% for those with partial hematologic remission or resistant disease. Only two of the 36 patients who achieved complete hematologic remission on IFN-α therapy have died, compared to 9 of the 15 patients who did not (6% v 60%; $P < 0.01$).

As with remission induction, different patterns of survival were observed in different risk categories: the 3-year survival

Table 6. Early and Late Toxic Reactions of IFN-α Therapy

Toxicity	No. Patients (Percentage)		
	Overall	Mild-Moderate	Severe
Early			
Fever	41 (80)	36 (71)	5 (10)
Chills	23 (45)	23 (45)	—
Bone pains	8 (16)	7 (14)	1 (2)
Fatigue	35 (69)	35 (69)	—
Nausea and vomiting	7 (14)	6 (12)	1 (2)
Anorexia	12 (24)	12 (24)	—
Myalgias	18 (35)	18 (35)	—
Headache	8 (16)	8 (16)	
Late			
Fatigue	24 (47)	23 (45)	1 (2)
Bone pains, arthralgias	19 (37)	7 (14)	12 (24)
Myalgias	17 (33)	14 (27)	3 (6)
Weight loss	21 (41)	17 (33)	4 (8)
Neurotoxicity frontal	9 (18)	6 (12)	3 (6)
Parkinsonism	2 (4)	1 (2)	1 (2)
Immune-mediated thrombocytopenia	4 (8)	2 (4)	2 (4)

was 100% in the low-risk group, 75% in the intermediate-risk group, and 42% among the high-risk patients ($P < 0.01$).

DISCUSSION

Interferons have well-established antiviral and antiproliferative activities.[13] They were shown to suppress the growth and differentiation of normal and CML myeloid progenitor cells in vitro[24] and to induce granulocytopenia among treated patients.[15,17,25] The excessive myeloid proliferation in CML,[26] the possible viral etiology of CML shown in animal models,[27,28] and the success of interferon therapy for chronic or indolent diseases (hairy cell leukemia, renal cell cancer, indolent lymphomas and myeloma)[15-17] led us to investigate the clinical activity of IFN-α in CML.

The administration of human leukocyte IFN-α in the current study resulted in complete hematologic remission in the majority of patients. Therapy affected the peripheral blood, bone marrow, and spleen. The process of response was gradual, requiring a median of 14 weeks of therapy to achieve a complete hematologic remission and a median of 9 months to observe initial Philadelphia chromosome suppression. Changes were seen first in the peripheral blood, while bone marrow changes appeared late. A cyclic pattern of clinical response was observed in about one quarter of patients, possibly due to preservation of a partial feedback mechanism.[29,30]

Since our study used strict criteria in defining complete hematologic remission, the complete hematologic response rate of 71% is not comparable to response rates reported for myleran or hydroxyurea. Studies with these agents often did not require disappearance of palpable splenomegaly or complete normalization of the peripheral differential counts.[6,7]

Most interesting was the gradual suppression of the Philadelphia chromosome among responding patients. Previous studies with intensive combination chemotherapy resulted in significant suppression of the Philadelphia chromosome, with 30% to 60% of patients showing a decrease of Philadelphia chromosome-positive cells to less than 35%, and 10% to 30% manifesting 100% diploid metaphases.[8,31,32] These "true" complete and partial remissions were transient in all patients.[8,31] This demonstrates the existence of cells with a normal karyotype in the marrow of patients with CML that could repopulate the patient's marrow after eradication of the leukemic clones.[33,34] In contrast to the results of intensive chemotherapy, suppression of the Philadelphia chromosome-positive clones with IFN-α was slow to occur, progressive in nature, and long-lasting in a substantial number of our patients (Table 4). Suppression of the Philadelphia chromosome was also more pronounced among low-risk patients. The dramatic but transient cytogenetic response patterns with intensive chemotherapy and the slowly developing but persistent ones with IFN-α may be enhanced and maintained by the use of a combined modality approach.

The cost and scarcity of the partially pure human IFN-α material have been a significant problem in the conduct of large-scale clinical trials; this has been avoided recently through the availability of the pure recombinant alpha interferons. It has been argued that response to IFN-α or its toxicity may be partly attributed to the impurities of this partially purified form of interferon alpha. However, our recent preliminary experience with the pure recombinant alpha A interferon has demonstrated similar response and toxicity patterns.[19] Of the first 17 patients treated, 13 (76%) achieved a complete hematologic remission, and six (35%) had evidence of suppression of the Philadelphia chromosome.[19]

The chronic course of CML disease requires long-term follow-up to evaluate treatment benefit. One important question is whether IFN-α therapy will change overall patient prognosis. In this CML population, the yearly risk of death of 6% to 9% in the first three years compares favorably with the expected yearly mortality of 25% after the second year of follow-up for patients treated with single-agent therapy with myleran or hydroxyurea.[4-7] The 76% projected survival at 3 years is also encouraging in comparison with the 3-year survival rate of 53% among a historic control group of 155 patients from our institution who were treated with hydroxyurea or myleran[10] and with the 3-year survival percentage range of 35% to 65% in other reports.[1,2,4-7,11] The yearly incidence of blast crisis ranged from 5% to 15% in the first 3 years (Fig 3) and is lower than previously reported.[4-7] Interestingly, the majority of patients who developed blastic crisis manifested lymphoid or undifferentiated cellular morphologic characteristics. This contrasts with the reported 60% to 75% incidence of myeloid blastic crisis morphology reported in CML. This raises the possible advantage of additive chemotherapy in suppressing involved lymphoid cells. Finally, response to IFN-α seems to be a good parameter for predicting long-term prognosis. The 3-year survival rate was 94% for patients having a complete hematologic response and 45% for the remaining population.

The study of prognostic factors and risk categories in CML is useful in several ways,[10,35,36] including therapeutic strategy planning. Early intensive chemotherapy was shown previously to benefit mainly patients in the intermediate- and high-risk prognostic categories.[10] The present analysis demonstrates a substantial benefit of IFN-α therapy among intermediate- and low-risk patients. The combined use of intensive chemotherapy and interferons in a common treatment program may improve the outcome of all risk categories.

The acute toxic reactions to IFN-α were moderate. Chronic toxicities were treatment-limiting in 12% of patients. Serious toxicities included skeletal, muscular, and joint pains, neurotoxicity, and immune-mediated thrombocytopenia. Of interest was the simultaneous development of severe toxicity and resistant disease in a number of patients, raising the possibility of the release of certain factors from granulocytes that could induce inflammation in the musculoskeletal system.

In summary, this study showed IFN-α to induce hematologic remission in a majority of patients with chronic-phase CML. There were marked associated changes in the blood and bone marrow including suppression of the Philadelphia chromosome-positive clones. The unique biologic activity of IFN-α in CML opens possibilities for studies with interferons alone and in combination with intensive chemotherapy in an attempt to delay the onset of blastic crisis and to improve survival in this patient population.

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An Analysis of Leukemic Cell Chromosomal Features in Infants

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Leukemic cell chromosomal findings in 27 infants were analyzed. Among the 18 cases of acute nonlymphoblastic leukemia (ANLL), all but two were classified as monocytic or myelomonocytic. The remaining nine cases were acute lymphoblastic leukemia (ALL), seven lacking the common ALL antigen and two having cytoplasmic immunoglobulin (pre-B phenotype). Twenty-five cases (93%) had an abnormal karyotype, 21 (84%) being pseudodiploid. Chromosomal translocations were detected in 67% of the ANLL cases and in 78% of the ALL cases. Nonrandom chromosomal abnormalities included the t(9;11)(p21-22;q23) in three cases of monocytic leukemia, inversion of chromosome 16 in three cases of myelomonocytic leukemia with bone marrow eosinophilia, and t(4;11)(q21;q23) in one case of ALL. Chromosomal regions preferentially involved in infant leukemia included 11q23-25 (13 cases), 9p21-22 (6) and 10p11–13 (3). All but one of the 24 cases with chromosomal breakage or rearrangement had breakpoints that corresponded to known fragile sites, half of which were at 11q23-25, a finding that may have pathogenetic importance. The CALLA$^-$ or pre-B phenotype and the presence of chromosomal translocations in most infants with ALL provide a biological explanation for their poor prognosis.

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THE CLINICAL and biological hallmarks of acute leukemia in infants differ markedly from those of older children. Infants with acute nonlymphoblastic leukemia (ANLL) generally have acute monoblastic or myelomonoblastic subtypes with a propensity for extramedullary involvement.[1,2] Acute lymphoblastic leukemia (ALL) in infants is usually characterized by a large leukemia cell burden at diagnosis and by a blast cell immunophenotype that lacks the common ALL antigen (CALLA).[3-5] Treatment outcome tends to be poor in infants with either form of leukemia.[3,4,6]

Chromosomal abnormalities in leukemia have attracted much recent attention. The specific karyotypic patterns not only provide important prognostic information and allow more accurate classification, but also may aid in understanding leukemogenesis. Despite the known unique features of infant leukemia, cytogenetic studies in these patients have been limited to occasional case reports.[7] Because investigators tend to report nonrandom chromosomal changes such as the t(4;11),[8,9] little attention has been paid to the exact karyotypic distribution in cases of infant leukemia. In a relatively large series of consecutive cases of infant leukemia, we observed a high frequency of chromosomal translocations, which could explain the extremely poor prognosis associated with ALL in infants. Furthermore, our data suggest an unusual pathogenesis for acute leukemia in infants.

MATERIALS AND METHODS

Patients. From February 1979 to May 1986, 37 children aged <12 months with newly diagnosed acute leukemia were admitted to St. Jude Children's Research Hospital. Twenty-seven cases had adequate bone marrow samples and successful chromosome banding studies and are the subjects of this report. There were 18 cases of ANLL and nine of ALL. Informed consent was obtained for all patients, and the investigation was approved by the institution's clinical trials review committee.

Chromosome analysis. Bone marrow samples, obtained at the time of diagnosis, were processed according to the method of Williams et al.[10] Marrow cells were collected in RPMI 1640 medium with L-glutamine, antibiotics, and 30% fetal calf serum. The cells were immediately exposed to colcemid (final concentration, 0.06 μg/mL), rinsed twice in Hanks' balanced salt solution, and exposed to hypotonic KCl (0.075 mol/L) for a total of 32 minutes at room temperature, including periods of mixing, standing, and centrifugation. They were then fixed in 3:1 methanol-acetic acid (vol/vol) for 15 minutes, and slides were prepared by a flaming technique. Appropriate drying of the slides was achieved by natural aging for zero to seven days. Metaphase preparations were G-banded by a modification of the trypsin method of Seabright.[11] Chromosomal abnormalities were classified according to the International System of Human Cytogenetic Nomenclature (1985).[12]

Blast cell phenotyping. Cases of leukemia were classified according to French-American-British (FAB) criteria, based on bone marrow cell morphological and cytochemical staining characteristics.[13] Cases of ALL were further subtyped according to their immunophenotypes, as described previously.[14]

RESULTS

The presenting features, karyotypes, and treatment responses of the 27 infants with analyzable chromosomes are shown in Table 1. With two exceptions, the FAB types in cases of ANLL were either monocytic or myelomonocytic. Leukocyte counts for patients with ANLL ranged from 5.1 to 751 × 10^9/L (median, 63 × 10^9/L), platelet counts from 15 to 510 × 10^9/L (median, 71 × 10^9/L), and hemoglobin levels from 3.6 to 11.4 g/dL (median, 9.7 g/dL). Initial CNS leukemia was evident in ten patients. Bone marrow eosinophilia was noted in patients 11, 14, and 15 (18%, 15%, and 10% eosinophils, respectively); the first two patients had both

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Table 1. Clinical and Laboratory Data of Infant Leukemia

Patient no.	Age	Phenotype	% Blast in Bone Marrow	Leukocyte Count ($\times 10^9$/L)	CNS Leukemia	Karyotype	Duration of Remission (mo)
Acute Nonlymphoblastic Leukemia							
1	2d	M4	25	751	No	46,XY,t(10;11)(p11;q23)	3
2	2m	M5	80	70.9	Yes	46,XY,t(10;13)(p?15;q14)	2
3	2m	M4	81	24.1	No	46,XY,inv ins t(6;11)(q24;q23q12)	0
4	2m	M2	25	103.6	Yes	46,XX	6+
5	2m	M5	25	7.1	No	46,XX	6+
6*	3m	M2	22	11.5	No	47,XY,+21,i(7q)	0
7	4m	M4	69	400	No	46,XX,t(9;11)(q34;q12)	30+
8	6m	M5	93	12.4	Yes	46,XY,−10,+der(10),t(10;?)(q22;?),del(11)(q23),t(12;16)(q13;q24)	18
9	6m	M4	72	79.6	Yes	46,Y,del(X)(q23),−14,+der(14),t(14;?)(q32;?)/ 46,Y,del(X)(q23),del(6)(q23),−14,+der(14),t(14;?)(q32;?)	24
10	6m	M4	86	91.7	Yes	46,X,t(X;11)(q13;q23)	3
11	8m	M4	33	117	Yes	46,XX,inv(16)(p13q22)	2
12	8m	M5	19	5.6	Yes	46,XY,t(9;11)(p22;q13)	47+
13	8m	M5	85	5.1	Yes	46,XX,t(9;11)(p21;q23)	47+
14	8m	M4	31	55.5	Yes	46,XX,inv(16)(p13q22)	6
15	9m	M4	58	336	No	46,XX,inv(16)(p13q22)	0
16	9m	M5	90	9.4	No	47,XX,+X,t(9;11)(p21;q23)	28+
17	9m	M5	94	105.8	No	46,XY,t(10;11)(p13;q21)	4
18	9m	M5	81	56	Yes	46,XY,t(9;11)(p21;q23)	5
Acute Lymphoblastic Leukemia							
19	1m	L1 CALLA$^-$	81	125.2	No	46,XX,t(10;11;19)(p11.2;q23;p13)	2
20	1m	— CALLA$^-$	64	110	Yes	46,XX,t(9;11)(p21;q23)	0
21	3m	L2 CALLA$^-$	98	201	Yes	46,XX,t(4;11)(q21;q23)	30
22	4m	L1 CALLA$^-$	100	75	No	46,XY,t(1;11)(p22;q23)	45
23	6m	L2 CALLA$^-$	90	106	No	46,XX,t(1;11;11)?(p34;p13;q24)	26+
24	6m	L1 CALLA$^+$, pre-B	88	90	Yes	46,XX,inv(14)(q22q31)	7
25	7m	L1 CALLA$^-$	97	84	Yes	46,XX,+19,−22,t(9;11)(p13;q23)	34
26	9m	L1 CALLA$^-$, pre-B	83	8.5	No	45,XY,−5,−9,+der(9),t(5;9)(q11;p21)	21
27	9m	L1 CALLA$^-$	89	18.4	No	47,XY,+8	14+

*Down's syndrome.

CNS leukemia at diagnosis and at relapse. Only six of these patients remain in remission, for 6+ to 47+ months.

CALLA$^-$, so-called undifferentiated (HLA-DR$^+$ only), ALL was diagnosed in seven infants and pre-B ALL (positive cytoplasmic immunoglobulin) in two, one of whom was CALLA$^-$. In case 20, two thirds of the blasts had L1 morphology, and the remainder resembled monoblasts, although the α-naphthyl butyrate esterase stain was negative. A lymphoid-associated antigen, B4, was present on 65% of blast cells and the myeloid-associated antigen My-9 on 39% of blasts. Leukocyte counts in cases of ALL ranged from 8.5 to 201 × 10^9/L (median, 90 × 10^9/L), platelet counts from 7 to 354 × 10^9/L (median, 42 × 10^9/L), and hemoglobin levels from 4.9 to 11.2 g/dL (median, 8.7 g/dL). Four infants had CNS leukemia at diagnosis; none had a mediastinal mass. Two of the nine patients remain in remission for 14+ and 26+ months.

Of the 18 cases of ANLL, 16 (89%) had leukemic cell chromosomal abnormalities (Fig 1). The most frequent structural abnormalities were translocations (12 cases, 67%), inversions (three cases), and deletions (two cases). Whole chromosome additions were noted in two cases, including patient 6 (a child with Down's syndrome); none of the cases showed a loss of whole chromosomes. The structural abnormalities most frequently involved chromosomes 11 (ten cases), 9 (five cases), 16 (four cases), and 10 (four cases). The chromosomal regions most commonly affected were 11q23-25 (seven cases), 9p21-22 (four cases), 11q12-13 (three cases), 16p13 (three cases), 16q22 (three cases), and 10p11-13 (two cases). Nonrandom chromosomal abnormalities included t(9;11)(p21-22;q23) in three cases of monocytic leukemia[15] and inversion at bands p13 and q22 of chromosome 16 in three cases of myelomonocytic leukemia with bone marrow eosinophilia.[16,17]

All nine infants with ALL had blast cell chromosomal abnormalities (Fig 2): pseudodiploidy in seven cases, hypodiploidy with 45 chromosomes in one, and hyperdiploidy with 47 chromosomes in one. Translocations were observed in seven cases (78%), of which six involved 11q23-25 and two involved 9p21.

In both ANLL and ALL, chromosomes 11, 9, and 10 were frequently involved in structural rearrangements, mainly translocations. The common breakpoints were 11q23-25 (13 of 27 cases, 48%), 9p21-22 (six cases, 22%), and 10p11-13 (three cases, 11%).

DISCUSSION

Chromosomal rearrangements involving 11q23-25 were the most frequent finding in this study of infant leukemia, affecting half of our patients. This contrasts with the much lower frequency of 11q23-25 rearrangements in consecutive cases of childhood ALL (4% of 230 cases) or ANLL (13% of

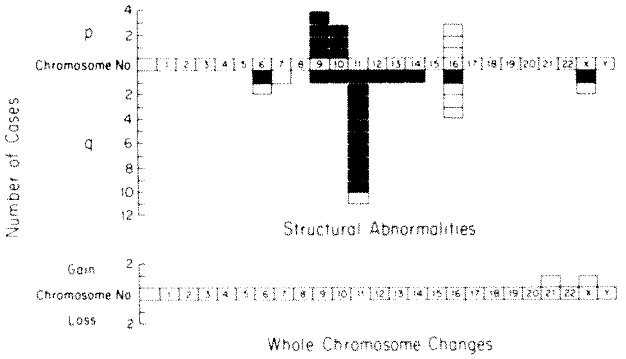

Fig 1. **Structural and numerical chromosomal abnormalities associated with acute nonlymphoblastic leukemia in infants. The closed blocks represent specific chromosomes involved in translocations and the open blocks all other abnormalities not associated with translocations. Each breakpoint is depicted once. The abnormalities primarily involved chromosomes 9, 10, 11, and 16. The added chromosome 21 was from a child with Down's syndrome.**

150 cases) seen at our institution (DL Williams and SC Raimondi, unpublished observation). Similarly, Abe et al[7] and Abe and Sandberg[18] noted a higher frequency of such abnormalities in case reports of infants with leukemia. The high proportion of cases with the t(4;11)(q21;q23) in their series probably reflects a reporting bias, as these cases have a poor treatment outcome and unique features including mixed lymphoid and myeloid antigen expression.[19,20]

The other frequent abnormality involving 11q23 was t(9;11)(p21;q23), a nonrandom abnormality that is known to be associated with monocytic or myelomonocytic leukemia,[15] the two commonest ANLL subtypes in infants.[1,2] Case 21 [ALL with the t(9;11)(p21;q23)] is interesting in that most of the blasts appeared to be lymphoid, with a small proportion resembling monoblasts. The findings of both B4 and My-9 positivity in blast cells support the mixed-lineage antigen expression of her leukemia. Kaneko et al[21] also reported this chromosomal abnormality in two cases of CALLA$^-$ ALL, one of which occurred in an infant. Thus t(9;11)(p21;q23) is not only found in monocytic or myelomonocytic leukemia but also in CALLA$^-$ ALL, including some cases with mixed-lineage antigen expression. Rearrangements involving 11q23-25 are in general common findings in CALLA$^-$ ALL.[21] Some cases of CALLA$^-$ ALL are characterized by myeloid-associated antigens[22]; in others the blast cells have been induced to differentiate in the myeloid pathway.[23] Taken together, these findings support the idea of preferential transformation of pluripotent stem cells with 11q23 involvement in infants and suggest that genes from the reciprocally translocated chromosome influence subsequent phenotypic expression.[18,21] Recently we found an 11q23 translocation at relapse in each of several cases that had converted from common or pre-B ALL at diagnosis to CALLA$^-$ ALL (three cases) or ANLL (three cases) at relapse.[24] We have suggested that these cases represent emergence of a malignant pluripotent stem cell after eradication of the original B-cell precursor or pre-B leukemic stem line by chemotherapy.

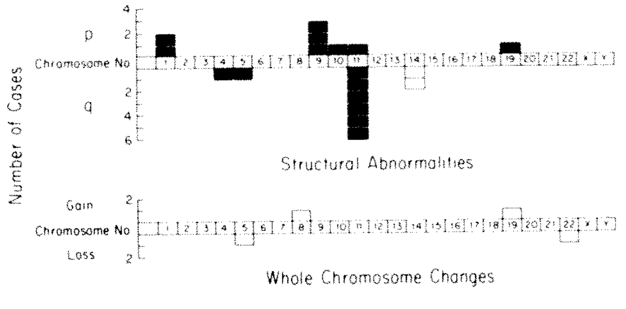

Fig 2. **Structural and numerical chromosomal abnormalities associated with acute lymphoblastic leukemia in infants. The closed blocks represent specific chromosomes involved in translocations and open blocks all other abnormalities. Each breakpoint is depicted once. Chromosomes 9 and 11 were frequently involved.**

Four of 29 cases of infant leukemia reviewed by Abe et al[7] had abnormalities involving chromosome 10, including three cases with a breakpoint at 10p11-13. Recently, another case of congenital monoblastic leukemia with del(10) (p12) was reported.[25] In this study four of 27 cases were found to have chromosomal abnormalities involving chromosome 10; in three cases the breakpoints were in the 10p11-13 region. By contrast, of 380 children with acute leukemia studied at this center, only five over a year of age had blast cell chromosomal rearrangement affecting this region ($P = 0.01$ by two-tailed Fisher exact test) (SC Raimondi and DL Williams, unpublished observation). Thus, the 10p11-13 region can be added to those known to be preferentially involved in infant leukemia.

The breakpoints in all but one of the 24 cases with chromosomal rearrangements (case 17) corresponded to known heritable or common fragile sites or both.[26-28] In 18 cases the breaks occurred at one or two heritable fragile sites, 13 being at 11q23 (FRA11B), five at 9p21 (FRA9A), three at 16q22 (FRA16B), and one each at 11q13 (FRA11A) and 12q13 (FRA12A). Confirmation of the putative association between heritable fragile sites and chromosomal breakpoints in infant leukemia will require further study of normal cells from the patients and their family members. Recently several studies have shown a concordance between heritable fragile sites and the breakpoints involved in chromosomal rearrangements in leukemia and lymphoma.[29-32] Hecht and Sutherland[33] found a statistically significant association between 21 fragile sites and 50 cancer breakpoints. It has been suggested that oncogenes or other very active genes may be located at or near hypersensitive or fragile sites where carcinogens can act, resulting in chromosomal breakage and rearrangement leading to malignant transformation.[27] Since infants are less likely than older patients to have experienced a carcinogenic event followed by a latency period adequate for the development of cancer, we speculate that acute leukemia in infants arises from constitutional or developmental genetic errors occurring at critical points in hematopoietic cellular ontogeny and differentiation.

Most of our cases had chromosomal breakpoint(s) where oncogene(s) have been mapped.[34] Since the c-*ets*-1 protooncogene has been traced to 11q23[35] and half of our cases have breakpoints in this region, it is possible that c-*ets*-1 is frequently involved in the pathogenesis of infant leukemia.

Rovigatti et al[36] have recently demonstrated amplification and rearrangement of this oncogene in a case of acute myelomonocytic leukemia and in a case of small lymphocytic-cell lymphoma with 11q23 involvement. It is also intriguing to note that α- and β-interferon genes have been localized to 9(p21 → pter),[37] a region commonly involved in infants with leukemia.

One of the most important prognostic factors in childhood ALL is age at diagnosis. Even after adjustment for other presenting features, a very young age remains an independent predictor of poor outcome.[38] However, in most studies leukemic cell chromosomal analyses have generally not been included, and their independent contribution to prognosis has not been assessed. In our most recent study,[39] the frequency of translocation was 40% among 116 consecutive children with newly diagnosed ALL. The high frequency (78%) of leukemic cell chromosomal translocations found in this study provides a biological explanation for the poor outcome of infants with ALL. Translocations in general appear to confer a poor prognosis in ALL.[40-42] The risk of early relapse was six times greater when a translocation was present, making this feature the strongest single predictor of outcome.[42] Notably, hyperdiploidy with >50 chromosomes, a favorable feature found in approximately 30% of childhood ALL cases,[39-44] was not found in any infant in this series or in the review by Abe et al.[7]

We conclude that most cases of infant leukemia are characterized by leukemic cell chromosomal abnormalities involving 11q23. The preferential involvement of 11q23 chromosomal region, the relationship between the breakpoints of reported fragile sites, and the likely minimal exposure of infants to environmental mutagens and carcinogens suggest a different mode of leukemogenesis in this young age group compared with older patients. Moreover, the poor outcome of infant ALL can be explained not only by their large leukemia cell burden and unfavorable immunophenotype but by the lack of leukemic cell hyperdiploidy and the frequent presence of chromosomal translocations. Finally, the definition of specific chromosomal anomalies in the blasts from infants with acute leukemia should provide direction for molecular studies that hold the exciting promise of unravelling the pathogenesis of these disorders.

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Measurement of Platelet-Associated IgG in Animal Models of Immune and Nonimmune Thrombocytopenia

By Janice Arnott, Peter Horsewood, and John G. Kelton

Platelet-associated IgG (PAIgG) is elevated in idiopathic thrombocytopenic purpura (ITP), but it also is elevated in other thrombocytopenic disorders traditionally considered to be nonimmune. Consequently it is possible that elevated PAIgG is a nonspecific finding secondary to thrombocytopenia. To study this issue we developed a rabbit model of immune and nonimmune mediated thrombocytopenia. The mechanism of the thrombocytopenia was validated by platelet survival studies. Immune thrombocytopenia was produced by injection of antirabbit platelet serum that was raised in guinea pigs. Nonimmune aregenerative thrombocytopenia was produced by irradiation of the animals; nonimmune consumptive thrombocytopenia was produced by injection of adenosine diphosphate (ADP). PAIgG was measured in a direct binding assay using ^{125}I-labeled staphylococcal protein A (SpA). Washed platelets from normal, nonthrombocytopenic rabbits bound an average of 81 molecules of SpA per platelet (81 ± 168, mean ± 2 SD, n = 39). Infusion of the antiplatelet antiserum produced thrombocytopenia with a rise in PAIgG that was closely correlated with the level of PAIgG (r = 0.86, n = 12). The thrombocytopenia was consumptive, as shown by a very short platelet life span using ^{111}In-labeled platelets. In contrast, both nonimmune thrombocytopenic states resulted in an equal or greater drop in the platelet count but no change in the level of PAIgG. The animals with aregenerative thrombocytopenia had normal or only moderately reduced platelet life spans; however, in every animal the level of PAIgG was not different from the nonthrombocytopenic controls, irrespective of the platelet count. Similarly, the level of PAIgG was unchanged in those rabbits with nonimmune consumptive thrombocytopenia following infusion of ADP (82 ± 55 molecules of SpA per platelet, mean ± SD, n = 6). These studies indicate that elevated PAIgG is a specific finding of immune thrombocytopenia and is not secondary to thrombocytopenia itself. Indirectly these results support our hypothesis that immune mechanisms contribute to more thrombocytopenic disorders than was once thought likely.

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FOR APPROXIMATELY 10 years, IgG on platelets has been measured qualitatively or quantitatively.[1,2] The amount of platelet-associated IgG (PAIgG) is elevated in about 90% of thrombocytopenic patients with idiopathic thrombocytopenic purpura (ITP).[3-6] This observation plus the demonstration that at least some of the IgG is specific for platelet glycoproteins[7] suggested that measurement of PAIgG could serve as a diagnostic test for ITP. However, PAIgG is also elevated in a large number of immune thrombocytopenic disorders, and consequently the test is not diagnostic for ITP.[8] Perhaps more importantly, elevated levels of PAIgG have been noted in a variety of disorders traditionally thought not to be caused by immune mechanisms.[8] These observations lead us to two postulates. First, they suggest that much of the IgG measured on platelets is not true autoantibody.[9] Second, the studies also suggest that immune mechanisms contribute to platelet destruction in more thrombocytopenic disorders than once was thought likely.[8]

It is difficult to study this issue in humans because many episodes of thrombocytopenia are associated with conditions such as sepsis and drug therapy, which by themselves might contribute to the thrombocytopenia.[8,10,11] For this reason we developed an animal model of three types of thrombocytopenia in rabbits: consumptive immune, consumptive nonimmune, and aregenerative nonimmune thrombocytopenia. When PAIgG was measured on washed platelets from animals with these various types of thrombocytopenia, we found that it was elevated in immune mediated thrombocytopenia but not in the nonimmune thrombocytopenias.

MATERIALS AND METHODS

Animal Studies

New Zealand white rabbits of either sex weighing 2 to 4 kg were used in all experiments. The animals were anesthetized with 30 mg/kg sodium pentobarbitol (MTC Pharmaceuticals, Hamilton, Ontario) administered intravenously (IV) through a marginal ear vein.

Preparation of Antiplatelet Serum

Antiserum against rabbit platelets was prepared in female guinea pigs. Whole blood from rabbits was collected into acid citrate dextrose (ACD, 6:1, vol:vol, pH 4.5), and the platelets were isolated by centrifugation, washed, and suspended in sterile saline. The guinea pigs were sensitized with an intraperitoneal (IP) injection of 10^9 platelets. Two further injections were given at one month intervals, and blood was collected, by cardiac puncture, at six days following the last injection. The serum was pooled and absorbed once (37°C for 60 minutes) with packed rabbit red cells (1:1, vol:vol) and once with washed rabbit lymphocytes. The serum was aliquoted and stored at −70°C until used.

The antiplatelet activity of the guinea pig serum was tested in vitro by two methods: enzyme-linked immunosorbent assay (ELISA) and radioimmunoprecipitation.

For the ELISA, rabbit platelets were isolated, washed three times, and resuspended to a concentration of 60,000/μL in 0.15 mol/L phosphate-buffered saline (PBS), pH 7.4. Fifty microliters of the platelet suspension was added to Immulon II (Dynatech, Alexan-

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dria, VA) wells and centrifuged at 560 g for 13 minutes. The wells were incubated with a blocking buffer (0.2% gelatin, 0.1% sodium azide, 0.05% Tween, 0.15 mol/L PBS) overnight at 4°C and washed. Fifty microliters of test serum was added, and the wells were incubated at 37°C for one hour. After washing, 100 μL of staphylococcal protein A alkaline phosphatase conjugate (Boehringer Mannheim Canada Ltd, Dorval, Quebec) was added and the wells incubated at 22°C for 45 minutes. After four washes, 200 μL P-nitrophenyl phosphate disodium (Sigma Chemical Co, St. Louis) at 1.5 mg/mL in diethanolamine buffer (Sigma) was added and after a 30-minute incubation at 37°C, the reaction was stopped by the addition of 50 μL of 1 N sodium hydroxide. The optical density (OD) of the solution was read at 405 nm using a Microplate Autoreader (Bio-tek Instruments, Burlington, VT). A result was defined as positive if the OD was greater than two times the background OD.

For the radioimmunoprecipitation assay, rabbit platelets were isolated from platelet-rich plasma (PRP) by centrifugation at 2,200 g for ten minutes and washed twice in 10 mmol/L Tris, 10 mmol/L EDTA, 150 mmol/L NaCl, pH 7.4. The platelets were resuspended at 10^9 per mL in the same buffer and labeled with 1 mCi Na ^{125}I per mL using IODO-GEN (Pierce Chemical Co, Rockford, IL). The labeled platelets were washed and then lysed in 1% Triton X-100 (Biorad, Mississauga, Ontario), 10 mmol/L Tris, 10 mmol/L EDTA, 150 mmol/L NaCl, pH 7.4 containing 100 μg/mL leupeptin (Sigma) and 0.1 mmol/L phenylmethylsulfonylfluoride (Sigma). Radiolabeled platelet lysate plus 50 μL of the guinea pig antiplatelet serum was incubated at 4°C for two hours and then added to 50 μL of protein A Sepharose CL-4B beads (Pharmacia, Dorval, Quebec) for 30 minutes. The beads were washed in lysing buffer and the bound proteins eluted by boiling for three minutes in 60 mmol/L Tris-HCl, 2% sodium dodecyl sulphate (SDS), pH 6.8. The supernatant was added to the stacking gel of a 5% to 20% gradient SDS-polyacrylamide gel and run at 10 mA for 20 hours. The gel was stained with Coomassie blue and dried for autoradiography. Labeled proteins were detected with Kodak XRP-1 film after 24 hours of exposure.

Animal Models of Thrombocytopenia

Consumptive immune thrombocytopenia. Animals were anesthetized and infused with increasing amounts of antiplatelet serum through the marginal ear vein. AT 15-minute intervals, whole blood was sampled through a carotid cannula into EDTA tubes (Becton Dickinson, Mississauga, Ontario), and platelet counts were performed. At 90 minutes blood was collected into acid-citrate-dextrose (ACD) for determination of PAIgG. Control animals were infused with either normal (nonimmunized) guinea pig serum (NGPS) or 0.9% sodium chloride. All platelet counts were performed on a model ZBI Coulter electronic counter (Coulter Electronics, Hialeah, FL).

Consumptive nonimmune thrombocytopenia. Consumptive thrombocytopenia was produced by the infusion of 100 mg/kg adenosine diphosphate (ADP) (Sigma) through the marginal ear vein. Blood for platelet counts and PAIgG determination was collected through a carotid cannula at ten minutes after infusion. This time was selected as preliminary studies demonstrated that the platelet nadir had occurred by ten minutes. Because the platelet nadir occurred within ten minutes, the effects of multiple ADP infusions were also tested. ADP (80 mg/kg) was infused once daily for four days, and the platelets were then tested for PAIgG following the last infusion.

Aregenerative thrombocytopenia. Aregenerative thrombocytopenia was produced by irradiation of the rabbits with a ^{137}Cesium source (γ-radiation) for 30 minutes. This exposure to 930 rad produced thrombocytopenia at five to seven days. Blood was collected as previously described.

Measurement of platelet-associated IgG. Platelet-associated IgG was measured in a direct binding assay using staphylococcal protein A (SpA). SpA binds with the same relative affinity to both rabbit and guinea pig IgG.[12] Whole blood was collected into ACD, the platelets were isolated, washed three times in 0.15 mol/L PBS, 0.013 mol/L EDTA, 0.2% bovine serum albumin (BSA), and the platelet count was determined. Duplicate samples of 100 μl of platelet suspension were incubated with 25 μL of increasing concentrations (0.125 μg/mL to 10 μg/mL) of ^{125}I-labeled SpA (Amersham, Oakville, Ontario). After incubation for one hour at 22°C with agitation, the platelets were carefuly layered onto silicon oil (Dow Corning, Newbedford, MA) of specific gravity 1.022 and centrifuged at 12,000 g for three minutes. The radioactivity of the platelet pellet was measured. To distinguish specific binding from nonspecific binding, the same binding study was repeated with a 50-times excess of unlabeled SpA (Sigma).

The number of molecules of SpA bound per platelet was calculated as follows: specific binding was defined as the saturable binding of ^{125}I-SpA that could be displaced by the 50-times excess unlabeled SpA. The radioactivity at saturation was converted to molecules of SpA using the total number of platelets and the specific activity of the SpA.

Indium-labeled platelet survivals. One hundred milliliters of rabbit blood was collected into ACD. The platelet pellet was obtained by centrifugation of the PRP, and the pellet surface was gently washed five times with calcium-free Tyrode's buffer, pH 6.2. The platelets were resuspended in calcium-free Tyrode's buffer plus ethylene glycol tetra-acetic acid (EGTA) and labeled with 180 μCi ^{111}Indium oxine (Amersham). The labeled platelets were washed twice and resuspended in 1 to 2 mL platelet-poor plasma. The radioactivity associated with platelets and any contaminating red blood cells was determined as follows: one hundred microliters of the final labeled suspension was added to each of 2 mL of 1% ammonium oxalate and to 2 ml of 0.9% sodium chloride, and the suspensions were centrifuged. The platelet-bound radioactivity was calculated from the ammonium oxalate pellet, and the total cell-bound radioactivity was determined from the sodium chloride pellet.

The labeled platelets were infused IV through the marginal ear vein of recipient rabbits, and blood samples (3 mL) were collected into ACD at 30, 60, and 120 minutes postinfusion and once daily for five to seven days. To estimate the platelet life span, the radioactivity of whole blood samples was measured and the data was analysed by computer best fit using gamma function analysis.

To perform platelet survivals in the immune thrombocytopenic animals, labeled platelets were infused as described, but at 15 hours postinfusion antiplatelet antiserum was injected, and blood samples were taken at 30, 60, 180, and 300 minutes after the serum injection.

Statistical analysis. The means of platelet counts in the animal models were compared using an unpaired Student's *t* test at the 0.05 significance level.

RESULTS

Measurement of Platelet-Associated IgG on Rabbit Platelets

The percent recovery of platelets through the silicon oil was validated using ^{51}Cr-labeled platelets. At the specific gravity of 1.022 used, greater than 95% of the platelets crossed the gradient. Over a platelet concentration of 60,000 to 1,000,000 platelets/μL, recovery was consistently greater than 95%.

Since ADP infusion may cause a decrease in the mean platelet density of circulating platelets, the passage of these platelets through oil was validated in the same way. Platelets from ADP-treated animals were labeled with ^{51}Chromium and passed through silicon oil (specific gravity, 1.022). Over a platelet concentration of 300,000 to 1,000,000 platelets/μL, recovery was equal to or greater than 99%.

Sizing experiments performed on ADP-treated platelets using a particle sizer and stained blood film showed them to be of normal size with no fragments.

Normal, nonthrombocytopenic rabbits bound 81 ± 168 molecules of SpA per platelet at saturation (mean ± 2 SD; n = 39 different rabbits). A representative binding curve is shown in Fig 1.

Platelet Survival Studies

The in vivo recovery of the radiolabeled platelets was defined as the percent radiolabeled platelets in the circulation at two hours postinfusion and was always greater than 75%. The mean platelet-associated radioactivity was 82%, and on average there was 4% red cell contamination. The average platelet life span of nonthrombocytopenic rabbits as determined by gamma function analysis was 68 ± 23 hours (mean ± SD; n = 15) (Table 1).

Consumptive Thrombocytopenia (Immune)

The antiplatelet activity of the serum was confirmed by the ELISA and the radioimmunoprecipitation assay. Normal (nonimmunized) guinea pig serum did not bind to rabbit platelets and gave a mean OD of 0.158 in the ELISA (n = 3). However, the immunized guinea pig serum reacted strongly with rabbit platelets, producing an OD of greater than 1.50 neat and an OD of 0.809 at a 1:128 dilution in buffer (n = 3).

The antisera was further tested using a radioimmunoprecipitation assay. The immune serum contained IgG antiplatelet antibodies that reacted with a large number of rabbit platelet glycoproteins as shown in Fig 2.

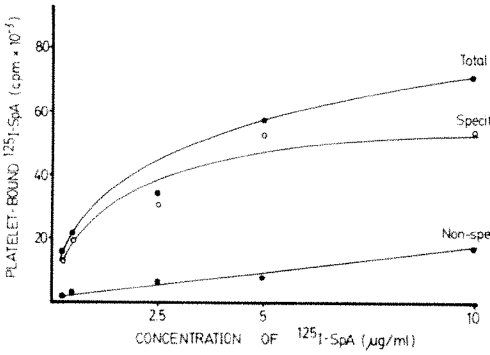

Fig 1. A typical binding curve for the PAIgG assay. Washed platelets from an antiserum-injected rabbit were incubated with increasing concentrations of ^{125}I-labeled SpA (abscissa), plotted against the radioactivity of the platelet pellet (ordinate). Specific binding is the saturable binding of ^{125}I-labeled SpA that is displaced by the 50-times excess unlabeled SpA and is calculated by subtraction of "background" nonspecific binding from total binding.

Table 1. ^{111}In-Oxine Platelet Survivals

Treatment of Rabbits	n	Platelet Life Span (hours)	Range (Hours)
Normals	15	68 ± 23*†	32.3-103
Aregenerative thrombocytopenia	10	30 ± 17*†	11.1-63.2
Consumptive immune thrombocytopenia	3	0.20	0.17-0.23

*Mean ± SD.

†Gamma function analysis.

Platelet counts and PAIgG determinations were performed on sham-treated (0.9% sodium chloride) rabbits and rabbits injected with normal guinea pig serum. In these animals the mean post-treatment platelet count was not significantly different from the mean platelet count of normal rabbits, and the PAIgG was within the normal range at a mean of 45 molecules of SpA per platelet (range 9 to 136 molecules SpA per platelet, n = 12).

Infusion of the antiplatelet antiserum produced an immediate consumptive thrombocytopenia. The mean platelet count for these animals differed significantly from that of normal, nonthrombocytopenic animals ($P < 0.05$), (Fig 3B). In every rabbit given the antiplatelet antiserum, the level of PAIgG rose to a mean of 2,400 molecules SpA per platelet (range 815 to 4,330 molecules SpA per platelet, n = 12), (Fig 3A). The rise in PAIgG and the fall in the platelet count were both strongly correlated with the amount of antiserum administered. The severity of the thrombocytopenia correlated significantly with the level of PAIgG (r = 0.86, n = 12), as shown in Fig 4A. Similarly, the level of PAIgG

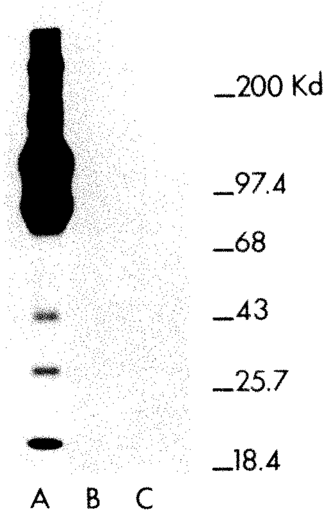

Fig 2. An autoradiograph of the radioimmunoprecipitation assay using ^{125}I-labeled rabbit platelets and the guinea pig antirabbit platelet serum. This serum reacted with a large number of rabbit platelet glycoproteins (lane A). Normal guinea pig serum (lane B) and saline (lane C) did not precipitate any proteins.

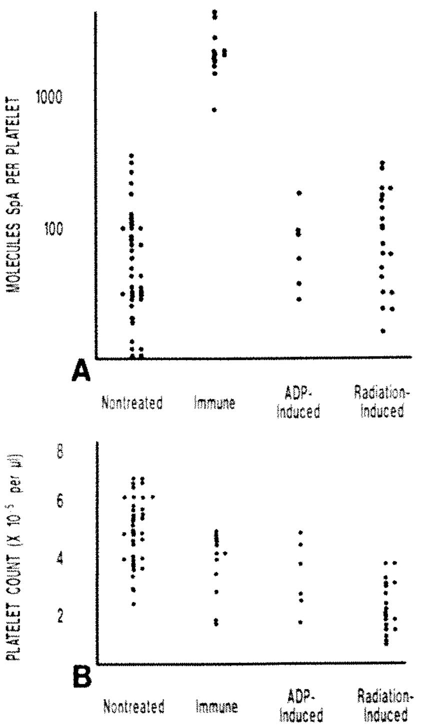

Fig 3. (A) Molecules of SpA per platelet in the immune and nonimmune thrombocytopenic animals. PAIgG, expressed as molecules of SpA per platelet, is plotted on semilogarithmic paper (ordinate) against nontreated and thrombocytopenic rabbits (abscissa). (B) Platelet counts in the immune and nonimmune thrombocytopenic rabbits. The platelet count (ordinate) is shown for nontreated and thrombocytopenic rabbits (abscissa). The mean platelet count for the nontreated rabbits is significantly higher than the mean platelet counts for the thrombocytopenic rabbits ($P < 0.05$).

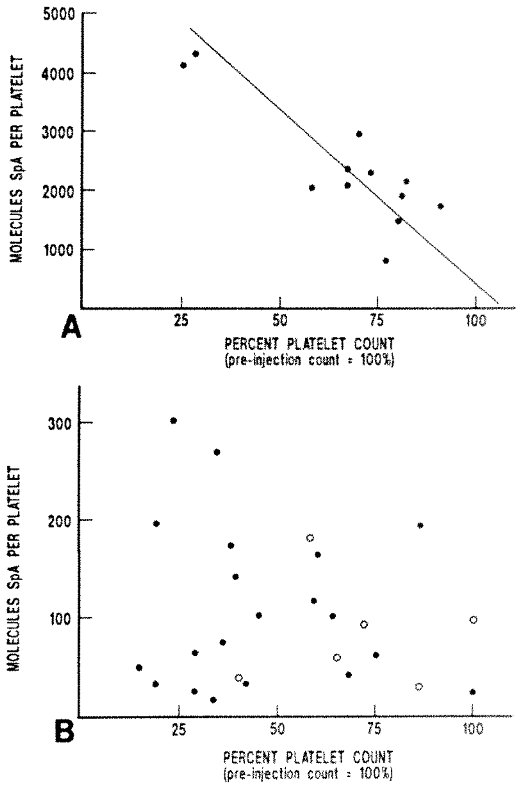

Fig 4. (A) PAIgG plotted against the platelet count in the immune thrombocytopenic rabbits. There was a significant correlation ($r = -0.86$, $n = 12$) between the PAIgG (ordinate) and the platelet count (abscissa). (B) PAIgG plotted against the platelet count in the ADP-treated (○) and irradiated (●) rabbits. There was no correlation between the PAIgG (ordinate) and the platelet count (abscissa) for the ADP-treated rabbits ($r = -0.02$, $n = 6$) or for the irradiated rabbits ($r = -0.13$, $n = 20$).

was directly correlated with the amount of antiserum injected ($r = 0.94$, $n = 12$).

Platelet survival studies were performed on the antiserum-injected animals. Following infusion of the antiplatelet antiserum, approximately 80% to 90% of the platelets were lost from the circulation within 30 minutes (Table 1).

Consumptive Thrombocytopenia (Nonimmune)

Infusion of ADP resulted in a significant fall in the mean platelet count ($P < 0.005$) in comparison with nontreated animals (Fig 3B). However, in contrast to the animals with immune thrombocytopenia, all of the rabbits with nonimmune consumptive thrombocytopenia caused by ADP infusion had levels of PAIgG that were within the normal range at 82 ± 55 molecules of SpA per platelet (mean ± SD, $n = 6$), (Fig 3A). In these animals there was no correlation between the platelet count and the level of PAIgG (Fig 4B). In the animals treated with multiple ADP infusions, the level of PAIgG remained within the normal range at 78 ± 26 molecules of SpA per platelet (mean ± SD, $n = 4$).

Aregenerative Thrombocytopenia

Irradiation of the rabbits resulted in platelet counts that were significantly lower than the mean for normals ($P < 0.0005$) (Fig 3B). In every one of these animals the PAIgG level remained within the normal range at 109 ± 84 molecules SpA per platelet (mean ± SD; $n = 20$), as shown in Fig 3A. In these animals there was no correlation between the platelet count and the level of PAIgG (Fig 4B).

The radiation-treated animals had a platelet life span that was normal or marginally reduced (Table 1).

DISCUSSION

Techniques for detecting IgG on platelets have been available for a number of years and have been used to investigate many different thrombocytopenic disorders. There is now general agreement that the level of platelet-associated IgG (PAIgG) is increased in about 80% to 90% of thrombocytopenic patients with idiopathic thrombocytopenic purpura.[2-4] The recent demonstration that antiplatelet antibodies in the serum of patients with ITP bind to platelet-specific antigens via the F(ab) terminus of the antibody molecule indicate that at least some of the IgG on the platelet surface represents true autoantibody.[7] However, a number of studies from our laboratory have raised several issues that are as yet unresolved. For example, our demonstration that the increase of platelet-associated IgG is paralleled by an increase in platelet-associated albumin suggests that much of the increased platelet-associated IgG is not true autoantibody.[9] In addition, platelet-associated IgG is elevated not only in typical "immune" thrombocytopenic disorders but also in many disorders traditionally not considered to be caused by immune mechanisms.[8] Taken together these observations have several possible interpretations: first, elevated levels of platelet-associated IgG might in part be due to artifacts of the test system, for example, contaminating platelet fragments. Recent studies suggest that this is an unlikely explanation.[13] Another possible reason for the elevated levels of platelet-associated IgG in nonimmune thrombocytopenic disorders is that the thrombocytopenia by itself could lead to elevated levels of platelet-associated IgG (the opposite of what is currently postulated). The studies described in this report were designed to address this issue. We studied animal models of thrombocytopenia because it is difficult to study "pure" thrombocytopenic disorders in humans. Typically, patients with one type of thrombocytopenia, for example, aplastic anemia, have thrombocytopenia that is complicated by other conditions, such as septicemia, which makes it difficult to isolate a single cause for the thrombocytopenia.

To investigate the relationship between the platelet count and the level of PAIgG, thrombocytopenia was produced in rabbits using several different techniques. The general mechanism responsible for the thrombocytopenia was validated with ^{111}Indium-labeled platelet survival studies. The life span of ^{111}Indium-labeled platelets in normal, nonthrombocytopenic rabbits was 68 hours (gamma function analysis), a finding similar to that reported by others.[14] Independently, the level of PAIgG was measured with a direct binding assay. The "positive control" was an animal model of immune thrombocytopenia. IgG antirabbit platelet antibody was raised in guinea pigs and was demonstrated by both ELISA and radioimmunoprecipitation (Fig 2) to react in vitro against a number of different glycoproteins. Following infusion of the antiserum into rabbits, a rapid destructive thrombocytopenia resulted, and greater than 80% of the ^{111}Indium-labeled platelets were cleared from the circulation within 30 minutes after infusion. The fall in the platelet count was strongly correlated ($r = 0.89$) with the amount of serum injected. Thus we were able to conclude that the binding of the antibody to the rabbit platelets was responsible for the thrombocytopenia. The amount of PAIgG on the rabbit platelets was measured with a direct binding assay using radiolabeled staphylococcal protein A (SpA) as the ligand. Staphylococcal protein A binds equally well to both rabbit and guinea pig IgG.[12] To measure PAIgG on washed rabbit platelets, the specific binding of the ^{125}I-SpA to the washed platelets was measured in the standard fashion with subtraction of background nonspecific binding from total binding. The amount of PAIgG was expressed as molecules of staphylococcal protein A per platelet because the binding ratio of the SpA to platelet-bound IgG was not determined. There was a relatively wide range in the level of PAIgG in the control rabbits (81 ± 168 molecules of SpA per platelet [mean ± 2 SD, $n = 39$; range of 10 to 361]). The range is similar to the range of PAIgG in healthy humans using a similar assay. The large range may reflect the difficulty in precisely measuring very low levels of PAIgG plus the large sample size ($n = 39$).

The infusion of the antiplatelet antiserum into the rabbits resulted in a significant fall in platelet count ($P < 0.05$) that was inversely correlated with the rise in the level of PAIgG. The level of PAIgG increased from an average of 81 molecules of IgG per platelet (normal rabbits) to a mean of 2,400 molecules of SpA per platelet (Fig 3A). The level of PAIgG was significantly correlated with the amount of antiserum injected ($r = 0.94$) and more importantly, with the severity of the thrombocytopenia ($r = 0.86$) as shown in Fig 4A. Sham-treated animals and animals administered nonimmunized guinea pig serum did not have a change in the platelet count or level of PAIgG.

The level of PAIgG was also measured in the animals with nonimmune destructive and nonimmune aregenerative thrombocytopenia. Nonimmune destructive thrombocytopenia was produced by the infusion of adenosine diphosphate (ADP) into rabbits. Other investigators have shown that ADP infusions result in a dramatic fall in the platelet count, presumably due to platelet sequestration within the lungs and microcirculation.[15] When the rabbits were administered ADP, the platelet count immediately fell to similar levels caused by the antiplatelet antibody. Yet, when the level of PAIgG was measured on the platelets from these rabbits, it remained within normal limits (Fig 3A), and there was no correlation between the severity of the thrombocytopenia and the level of PAIgG (Fig 4B). To exclude the possibility that nonimmune thrombocytopenia of a longer duration might product a rise in PAIgG, ADP was infused daily into rabbits, and the platelet-bound IgG was measured on day 4. The level of PAIgG remained within normal limits.

Aregenerative thrombocytopenia was produced by irradiation of the rabbits. This treatment results in destruction of stem cells and megakaryocytes, and the platelet count fell to an average of 50% of the pretreatment platelet count at five to eight days following irradiation. There was considerable variation in the life span of platelets in these animals ranging from normal (63 hours) to moderately shortened (11 hours) with the overall mean being 30 hours (Table 1). The reason for the shortened platelet life span in irradiated animals is uncertain but could be due to "fixed" platelet requirements, as has been postulated by one group of investigators.[16] Alternatively, infection could have caused some degree of platelet destruction; however, this hypothesis is unlikely, as the animals appeared healthy and blood cultures in four animals were negative for pathogens. Regardless of the precise cause for the slightly shortened platelet life span in these animals, the severity of the thrombocytopenia was equal to or more severe than the thrombocytopenia in the animals given the antiplatelet serum, yet in no irradiated animal did the level of PAIgG rise.

In none of our studies were we able to demonstrate any correlation between the severity of the thrombocytopenia and the level of PAIgG except in those animals whose thrombocytopenia was caused by immune mechanisms (Fig 4). These studies indicate that thrombocytopenia by itself is no associated with elevation in the level of PAIgG. However, it should be noted that we were only able to produce moderate thrombocytopenia in the animals, and it is possible that if the animals became extremely thrombocytopenic, slight elevations in the level of PAIgG might have been observed. Against this hypothesis is the demonstration that the fall in the platelet count in the nonimmune thrombocytopenic animals was equal to or greater than that achieved with the immune thrombocytopenic animals. Yet in contrast to the markedly elevated levels of PAIgG in the immune thrombocytopenic animals, there was absolutely no change in the level of PAIgG in the nonimmune thrombocytopenic animals. Looking for further evidence against the possibility that more severe thrombocytopenia might have "unmasked" a slight elevation in PAIgG, we carefully examined the level of PAIgG in those animals with the most severe nonimmune thrombocytopenia (Fig 3). In none of these animals was there even a trend toward increased levels of PAIgG.

In summary, these studies indicate that elevated levels of PAIgG are specific findings of immune thrombocytopenia and are not observed in animals with well-characterized nonimmune thrombocytopenia. Taken in conjunction with previous studies in humans, the current investigations support our hypothesis that immune mechanisms contribute to more thrombocytopenic disorders in humans than was once thought likely.

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Immature Dense Granules in Platelets From Mice With Platelet Storage Pool Disease

By Madonna Reddington, Edward K. Novak, Edward Hurley, Chitra Medda, Michael P. McGarry, and Richard T. Swank

Mepacrine uptake into platelets and bone marrow megakaryocytes was analyzed to further characterize the dense granule defects in a group of seven mouse pigment mutants that have characteristics of platelet storage pool disease (SPD). In contrast to our previous studies using electron microscopy, this method revealed that all mutants had normal numbers of dense granules. However, total mepacrine uptake in all mutant platelets was significantly diminished to less than 50% of normal uptake. Also, the flashing phenomenon observed when normal dense granules are irradiated with ultraviolet light was either greatly diminished or absent when platelets of individual mutants were similarly irradiated. Therefore the principal defect in the mutant platelets is an inability to accumulate dense granule contents rather than an absence of the granules. Mepacrine uptake into megakaryocytes was indistinguishable in normal and mutant mice. This indicates the mutant dense granule defects appear either very late in megakaryocyte development or early in platelet formation in correlation with development of the mature dense granule. By standard transmission electron microscopy we have not been able to detect gross structural or subcellular abnormalities in either platelets or megakaryocytes of mutant mice. It appears all seven mutants produce immature or functionally abnormal dense granules.

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SEVEN (beige, pearl, pallid, maroon, ruby-eye, light ear, and pale ear) separate recessive mutations of the mouse, which cause diluted pigmentation, have been shown to have associated increased bleeding times together with platelet storage pool disease (SPD).[1] The beige mouse[2] is an animal model for human Chédiak-Higashi syndrome (CHS),[3] and the remaining mutants are candidate animal models for other human platelet storage pool diseases such as Hermansky-Pudlak syndrome (HPS).[4,5] The symptoms of human platelet storage pool deficiency have been subclassified into four groups.[6] This suggests human SPD, like mouse SPD, may be caused by different genetic loci.[6]

These seven mouse pigment mutants have defects in structure and/or function of at least three subcellular organelles. First, melanosomal abnormalities in several cases are responsible for their diluted pigmentation.[7] Second, all have abnormal secretion of lysosomal enzymes from kidney proximal tubule cells.[8,9] Also, when platelets are stimulated with thrombin, abnormal lysosomal enzyme secretion is observed in beige, pearl, pallid, light ear, and pale ear.[1] Similarly, when macrophages of pale ear mice are treated with amines, lysosomal enzyme secretion is reduced.[10] A well-documented presence of giant lysosomes occurs in many tissues of the beige mouse[11] as well as in patients with CHS syndrome.[3] Ceroid-like deposits observed in kidneys of mouse models of HPS[12] have recently been described in an autopsy of a HPS patient.[13] The third defective organelle in all seven mouse pigment mutants is the platelet-dense granule. Platelets from all seven mutants have abnormally low dense granule contents such as adenine nucleotides and serotonin.[1,14] Also, by electron microscopic analyses of whole-mounted platelets, very few dense granules are visible in any mutant platelets, and most platelets have no dense granules.[1,14] Thus these mouse pigment mutants emphasize the interrelatedness of different subcellular organelles and represent a rich source of separate genes that control the biogenesis, structure, and/or processing of these organelles.

By reciprocal bone marrow transplantation,[15,16] we have shown that in all seven mouse models of platelet storage pool deficiency the bleeding defect is likely due to an intrinsic defect expressed in marrow-derived platelet progenitor cells. To determine the stage in platelet or megakaryocyte development in which the dense granule defect first appears, the fluorescent dense granule marker, mepacrine, was used. This basic compound provides a useful method of monitoring dense granules of platelets and megakaryocytes, since it is rapidly and specifically concentrated in dense granules.[17,18] We report our analyses of dense granules of platelets and megakaryocytes of normal and seven separate SPD mouse mutants using mepacrine labeling. The results obtained using animal models for SPD are compared to those described in patients with CHS and HPS.

METHODS

Animals. Normal C57BL/6J and congenic or coisogenic homozygous mutants—beige (*bg/bg*), pearl (*pe/pe*), pallid (*pa/pa*), light ear (*le/le*), pale ear (*ep/ep*), maroon ($ru\text{-}2^{mr}/ru\text{-}2^{mr}$), and ruby (*ru/ru*)—were either obtained from the Jackson Laboratory, Bar Harbor, ME, or were bred in our animal facilities. Animals approximately 2 to 4 months of age and of both sexes were used. The properties of these pigment mutants, which exhibit symptoms of platelet storage pool diseases, are reviewed by Novak et al,[1] and their history is reviewed by McGarry et al.[16]

Isolation of platelets and megakaryocytes. Animals were killed by anoxia with CO_2. Blood was collected by heart puncture into a 22-gauge needle attached to a 1-mL syringe containing 0.1 mL 3.8% sodium citrate.[19] Platelet-rich plasma was isolated by centrifugation ten minutes at 150 g.[20]

Megakaryocytes were flushed from each femur with 0.75 mL "mega" medium containing 1 mmol/L adenosine, 2 mmol/L theophylline, 18.7 mmol/L Hepes buffer, 100 mmol/L glycine, 5 mmol/L EDTA, and 5% fetal calf serum in calcium and magne-

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sium-free Hanks' balanced salt solution, pH 7.0, containing 40 U/mL of DNase.[21] The bone marrow cell suspension (3 to 5 × 10^8 cells/mL) was filtered through a wire 200 mesh filter, then partially purified first by Percoll gradients followed by Ficoll density gradient (2% to 5% Ficoll) centrifugations according to Rabellino et al.[22] In later procedures the Percoll gradient fractionation was eliminated, since the percent purification was unchanged and yield was improved. Filtered bone marrow cell suspension (1.5 mL) was layered onto a 12.0 mL 2% to 5% Ficoll gradient and spun at 100 g for ten minutes. The pellet was resuspended in 1.0 mL mega medium, layered onto a second 6-mL 2% to 5% Ficoll gradient, and centrifuged at 100 g for ten minutes. The pellet was resuspended in 1.0 mL mega medium and used immediately. Samples were stained for acetylcholinesterase (specific for rodent megakaryocytes and precursors,[23] to monitor the purification. Typically, partially purified preparations contained 10% megakaryocytes. In control experiments it was found that both small and large bone marrow cells accumulated mepacrine in granules. To selectively analyze megakaryocytes for mepacrine uptake, only cells larger than 20 μm in diameter were monitored. Analysis of partially purified megakaryocyte preparations revealed that 60% of acetylcholinesterase-positive cells had diameters larger than 20 μm, whereas 90% of acetylcholinesterase-negative cells had diameters less than 20 μm. Thus this procedure ensured that we were preferentially monitoring uptake into megakaryocytes, although it did not allow measurements on smaller (<20 μm) megakaryocytes.

Mepacrine uptake. Platelet-rich plasma containing 5 × 10^{-5} mol/L mepacrine was incubated for 30 minutes at 37°C. Platelets were harvested by centrifugation at 1,000 g for ten minutes, washed twice with Tyrode's calcium-free solution,[1] and resuspended in the above Tyrode's solution at a 3 to 5 × 10^8 cells/mL. A drop of platelet suspension was placed on a glass slide with coverslip. After five minutes cells were analyzed in a Leitz MPV-2 fluorescent microscope with photomultiplier attachment. A mercury lamp (Osram model HBO, 200W/L2) with excitation filter at 450 to 490 nm and emission filter passing wavelengths above 510 nm was used. Platelets and megakaryocytes were analyzed under oil at 1,000 × magnification. Extraneous light was minimized by centering cells in the middle of a constricted circle formed by an iris diaphragm. The number of granules in individual cells was first counted. After a few seconds the granules began flashing. Both individual granules and granule flashes were recorded visually. Light photomicrographs of platelets were obtained in separate experiments with Kodak Tri-X Pan 400 film using the same microscope by exposing the cells to ultraviolet light for only five seconds to avoid the flashing phenomenon that occurs upon prolonged exposure to ultraviolet light.

Intensity of fluorescence (millivolts) was determined in separate platelets with a MPV-2 Leitz-Wetzlar photomultiplier together with recorder. To avoid discharge of granule contents by prolonged irradiation with UV light, the illuminated field was changed after examination of each cell. Standard curves with known concentrations of mepacrine in solution over the entire range of intensities recorded in platelets and megakaryocytes verified that the response was linear.

Electron microscopy. Platelets and partially purified megakaryocytes were prepared for electron microscopy according to White.[24,25] Specimens for electron microscopy were initially fixed in 0.1% glutaraldehyde in White's buffer and washed in buffer before fixation in 3% glutaraldehyde in Sorensen's phosphate pH 7.3 or White's buffer. They were rinsed in phosphate buffer, postfixed in 1% Osmium tetroxide, and embedded in Epon-Araldite. Sections were cut on an LKB III ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Siemens 101 electron microscope. Samples from two normal and two of each of the mutant mice were analyzed.

RESULTS

Mepacrine accumulation in platelets. Mepacrine was observed (Fig 1) to accumulate in discrete granules in normal and mutant platelets. Platelet cytoplasm contained only diffuse weak fluorescence. Photographs of platelets of three mutants—beige, pallid, and pale ear—are presented, but qualitatively similar results were obtained in all seven mutants of this study. Granules in normal platelets were more intense (see below) than in mutants, indicating an increased capacity to accumulate mepacrine.

When the number of platelet granules that accumulate mepacrine was enumerated (Table 1), there was in fact no significant difference between normal platelets and platelets from each of the seven mutants. All had five to six dense granules on average per platelet. One possible explanation of this result was that lysosomes were being counted as dense granules in mutant platelets. Mepacrine is an amine that is classified as a weak base. Weak bases are known to accumulate in lysosomes in several cell types.[26,27] To test whether mepacrine was taken up into lysosomes in addition to dense granules, we took advantage of the fact that both normal and beige platelets take up and secrete serotonin from dense granules after thrombin stimulation, while only beige platelets are selectively inhibited in lysosomal enzyme secretion.[1] Thus if mepacrine were taken up primarily into dense granules, mepacrine-containing granules would be equally and efficiently secreted on thrombin stimulation from both normal and beige platelets. If lysosomes were involved, normal platelets would secrete more mepacrine-containing granules than beige platelets. When thrombin-treated platelets were examined by light microscopy, both normal and beige platelets secreted all mepacrine-containing granules, indicating that in mouse platelets mepacrine accumulates primarily in dense granules. Skaer et al[18] have likewise presented evidence that mepacrine stains the dense bodies of platelets, not platelet lysosomes.

When an individual platelet was illuminated with ultraviolet (UV) light, discrete mepacrine-containing granules were observed. However, within a few seconds the granules began to flash and subsequently to fade. The flashing phenomenon, while incompletely understood, is thought to be a qualitative measure of the physical/chemical environment within the dense granule.[17] Illumination with UV light probably causes release of mepacrine into cytosol where it is less quenched, thus resulting in "flashing." It is apparent (Table 1) that there are marked differences in the flashes/platelet in normal and mutant mice. In normal mice the flashes per platelet are identical to the number of dense granules per platelet, indicating each dense granule flashes. In contrast, platelets of beige, pearl, and pallid mice exhibit little or no flashing (3% to 6% normal values). Higher rates of flashing were apparent in the light ear and pale ear mutants (43% and 37%, respectively, of normal) and the ruby-eye and maroon mutants (25% and 17% of normal). Thus in each of the mutants the number of flashes/granule is reduced, although the number of mepacrine-containing granules is normal.

Mepacrine accumulation during a 30-minute incubation was measured as the relative fluorescence intensity/platelet.

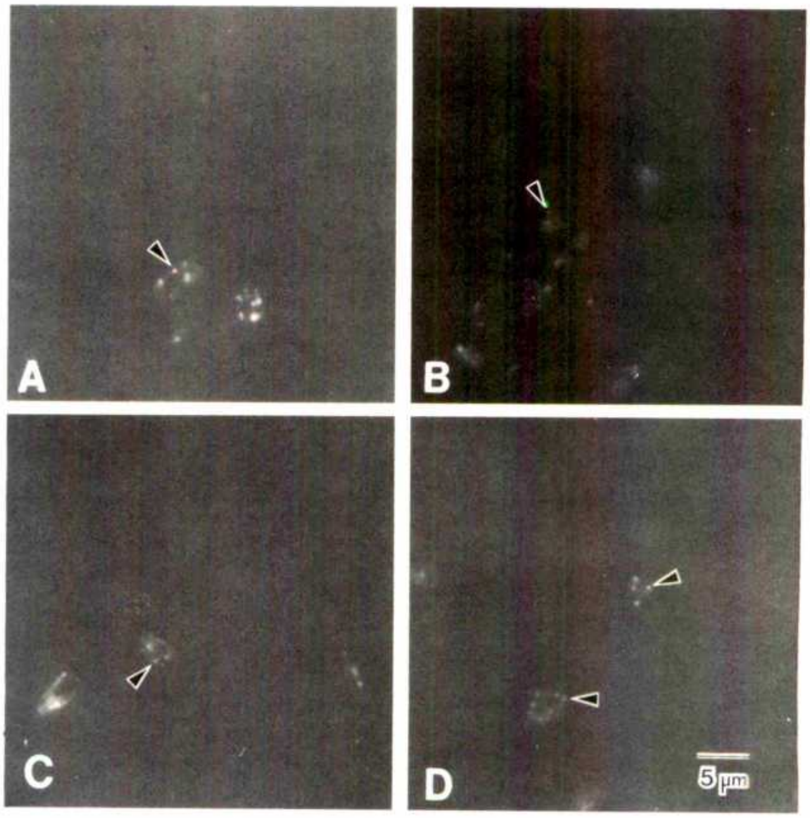

Fig 1. Visualization of dense granules in mepacrine-labeled platelets. Platelets from normal and mutant mice were treated with mepacrine, washed, and observed in a fluorescent microscope and photographed. In both normal and mutant platelets, mepacrine was localized in discrete granules (arrows). (A), normal; (B), beige; (C), pallid; (D), pale ear.

Platelets from all mutants accumulated less mepacrine than normal platelets. Of interest is the fact that the beige, pearl, and pallid mutants that were most abnormal in regard to the flashing phenomenon also accumulated the least mepacrine, only 19%, 16%, and 17%, respectively, of normal platelets. In the remaining four mutants, mepacrine accumulation was between 27% and 50% of normal accumulation.

Mepacrine uptake into megakaryocytes. Mepacrine uptake into partially purified large (>20 µm) megakaryocytes of bone marrow of normal and mutant mice was quantitated by fluorescence (Table 2). No significant difference in intensity was noted between normal and mutant cells. The variation in intensity within normal or mutant megakaryocytes was large. Plots of intensity *v* size (not shown) showed that this variation in mepacrine uptake was not correlated with cell diameter. It was also noted that the intensity of all megakaryocytes per unit cell volume was much less than that of platelets (compare Tables 1 and 2) given the fact that megakaryocytes may give rise to about 4,000 to 8,000 platelets.[28]

Although individual mepacrine-containing organelles were clearly visible in normal and mutant megakaryocytes, we did not attempt to count them because it was not possible to visualize all organelles in one focal plane in the relatively thick megakaryocytes. A significant finding was that flashing of mepacrine-labeled granules was observed in a small number (~1%) of normal megakaryocytes but was never observed in megakaryocytes from any of the seven mutants.

Electron microscopic analysis of normal and mutant platelets and megakaryocytes. Standard transmission electron microscopy of osmium-fixed thin sections revealed no gross structural abnormalities in mutant platelets (Figs 2

Table 1. Mepacrine Uptake in Platelet Granules

	Granules/Platelet	Flashes/Platelet	Flashes/Granule	Relative Intensity/Platelet (mV)
Normal C57BL/6J	6.2 ± 0.54	6.0 ± .69	0.99	73 ± 7.2 (100)
Beige	6.2 ± 0.57	0.2 ± .09*	0.03	14 ± 3.3* (19)
Pearl	5.0 ± 0.39	0.4 ± .12*	0.08	12 ± 4.2* (16)
Pallid	5.2 ± 0.69	0.3 ± .25*	0.06	12 ± 3.0* (17)
Light ear	6.0 ± 0.42	2.6 ± .30*	0.43	28 ± 3.8* (38)
Pale ear	6.3 ± 0.60	2.2 ± .39*	0.35	20 ± 8.0* (27)
Ruby-eye	5.9 ± 0.43	1.5 ± .32*	0.25	37 ± 2.8* (50)
Maroon	5.2 ± 0.47	1.0 ± .24*	0.19	30 ± 7.4† (41)

Characteristics of dense granules of normal and mutant platelets as revealed by mepacrine uptake determined by fluorescence microscopy. Values represent the mean ± SEM of 20 to 25 platelets in each case. Relative intensity/platelet was determined prior to commencement of the flashing phenomenon. Values in parentheses are the % intensity compared to the intensity of C57BL/6J platelets, normalized to 100%.

*$P \leq 0.001$.

†$P \leq 0.01$.

Table 2. Mepacrine Uptake Into Megakaryocytes

	Relative Intensity (mV/cell)
Normal C57BL/6J	6900 ± 740 (28)
Beige	5320 ± 940 (36)
Pearl	4960 ± 540 (65)
Pallid	5980 ± 620 (30)
Light ear	4340 ± 660 (40)
Pale ear	6300 ± 720 (30)
Ruby-eye	6460 ± 720 (50)
Maroon	6360 ± 880 (40)

Quantitative fluorescence values represent the mean ± SEM of the indicated number (in parenthesis) of megakaryocytes.

and 3). Although for comparison we have presented only pictures of normal and pallid platelets, all seven mutants and normal cells gave indistinguishable profiles for each cell type. All mutant cells had normal size and shape. Mutant platelets displayed the prominent circumferential ring of microtubules and appeared to have a normal intracellular complement of granules, mitochondria, and glycogen particles, and these organelles displayed a normal morphology. We did not attempt to analyze dense granules by this method. Platelet-dense granules are rarely seen in thin sections and are difficult to distinguish unequivocally from other platelet granules in these sections. Similarly, electron microscopic analysis (not shown) of megakaryocytes from normal and mutant mice revealed no gross structural abnormalities.

DISCUSSION

A major conclusion of this study is that all seven mouse pigment mutants, previously characterized as animal models of platelet storage pool disease and as having very few dense granules by electron microscopic analysis of unfixed, whole-mounted platelets[1] actually contain normal numbers of immature dense granules. The mutant platelet granules, however, are qualitatively abnormal. They accumulate much less mepacrine than normal granules and exhibit fewer flashes. In both of these properties, the mutants are divisible into two major groups. The beige, pearl, and pallid platelets have a near absence of flashes and less than 20% normal mepacrine accumulation, while light ear, pale ear, ruby-eye, and maroon platelets flash at 17% to 44% the normal frequency and accumulate 27% to 50% the normal mepacrine concentrations. In previous experiments[1] it was likewise found that the mutants were separable into the same two groups when concentrations of platelet-dense granule contents such at ATP and serotonin were examined. A likely explanation for the correlation between lowered mepacrine accumulation and decreased platelet-dense granule contents is that mepacrine has been shown to bind with high affinity to dense granule components such as ATP.[27] The lowered accumulation of mepacrine in mutant platelets is in agreement with the previously determined[1,14] lowered rate of accumulation of another dense granule component, serotonin, in the pearl, pallid, light ear, and pale ear mutants. Taken together the data suggest platelets from all seven mutants have normal numbers of immature platelet-dense granules that are unable to retain normal quantities of dense granule components.

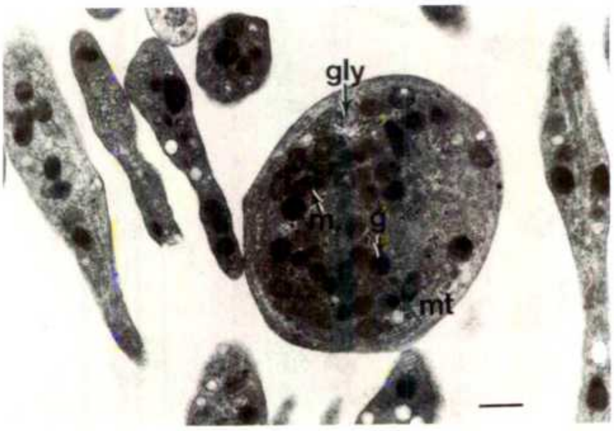

Fig 2. Analysis of platelets from normal mice by transmission electron microscopy. Abbreviations: m, mitochondrion; mt, microtubule; g, granule; gly, glycogen granules. Measuring bar = 0.5 μm.

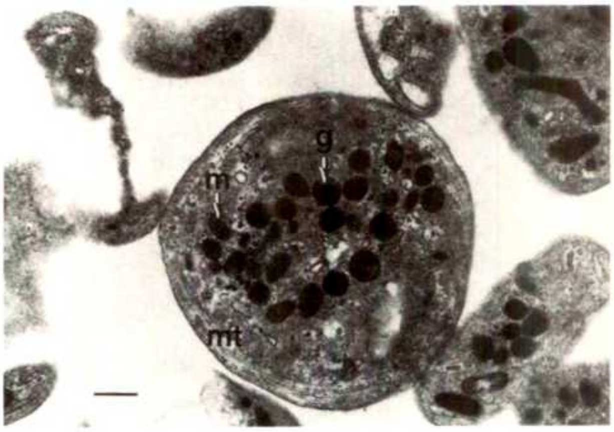

Fig 3. Analysis of platelets from pallid mice by transmission electron microscopy. Abbreviations: m, mitochondrion; mt, microtubule; g, granule. Measuring bar = 0.5 μm.

The results of these experiments can be compared to studies on various human and animal platelet storage pool diseases. In humans the two most studied forms of storage pool disease are Chédiak-Higashi (CHS) syndrome[3] and Hermansky-Pudlak syndrome (HPS)[4,5]

Several studies[11,29-31] of CHS platelets have demonstrated reduced levels of dense granule contents such as serotonin, ADP, and ATP. Most[29,32] but not all[11] morphological analyses of human CHS platelets have revealed decreased numbers of dense bodies.

In platelets from animal models of CHS storage pool disease,[1,14,19,29,33] symptoms similar to that of human CHS occur. Morphological studies by transmission electron microscopy using White's method[34] and the uranaffin fixation method[35] revealed a marked reduction of dense granules per platelet in cattle,[34] mink,[34] cat,[34] and beige mouse[35] models of CHS. However, when mepacrine labeling was used, only a small (35%) reduction of dense granules was observed in the beige mouse model.[36] In addition, similar to our results, the relative intensity of mepacrine accumulation was one-half normal, and flashing of beige platelet granules was virtually absent.[36]

Several groups have studied platelet dense granules in HPS. By morphological studies using White's method[37] and the uranaffin method,[38] dense granule numbers were greatly reduced in platelets from HPS. By mepacrine labeling using platelets from the same patients as the uranaffin method,[38] 50% to 70% of normal platelet-dense granules were found, and fluorescence intensity was 15% to 42% of normal. Flashing was measured in one patient and found to be 10% of normal levels.[38] Mepacrine studies with platelets from the fawn-hooded rat, an animal model for HPS, revealed 86% the normal dense granule number, 81% normal intensity, and 23% normal flashing. These results are similar to those obtained for the pale ear, light ear, ruby-eye, and maroon mutants.

We believe it is likely the studies[29,34,35,37,38] reporting large decreases in numbers of platelet-dense granules in CHS and HPS reflect limitations of the methods used. Two laboratories[34,37] used White's method[24,25] to analyze thin sections, others[35,38] the uranaffin method,[35] and still another[29] the whole-mount electron microscopic method.[39] These techniques depend upon having normal or near normal concentrations of dense-granule contents for identification in the electron microscope. This is true for the whole-mount method, which uses no fixatives and relies upon the natural density of the granule, and for the uranaffin method, which depends upon reaction with ATP in the granule. In White's method external calcium is included in the fixation medium to enhance dense granule contrast, but it is uncertain if this procedure adequately labels all dense granules in SPD patients. It is commonly found that other externally added components, such as serotonin and mepacrine, accumulate in dense granules of SPD patients to a much lower degree than in dense granules of normal patients. On the other hand, the mepacrine-labeling method utilized to study SPD platelets in this study and others[36,38,40,41] is a sensitive fluorescent method that is easily capable of revealing dense granules, even if only 15% to 20% the normal concentration is taken up into dense granules. We have analyzed the same normal and mutant mouse platelets by the mepacrine (this report) and whole-mount method.[1] It was found that the latter method revealed greatly reduced numbers of dense granules, while the mepacrine technique indicated normal dense granule number. Similarly, Richards et al[35] and Lorez and Da Prada[36] analyzed platelets of beige mice by the mepacrine and uranaffin methods and found no dense granules by the latter method but near normal numbers by the former. Rendu et al[32] analyzed normal and CHS platelets by the mepacrine technique and by White's method. While both methods revealed decreased contents of dense granules in CHS patients, the decrease was much larger with White's method. Thus it appears likely that the whole-mount, uranaffin, and White's methods are well suited for revealing "full" granules, while the mepacrine-labeling method detects both "full" and "empty" granules. We believe, therefore, that taken together, the available data suggest that while there is a partial reduction of platelet-dense granules in certain cases,[32,41] the platelet-dense granule defect in SPD is due to major qualitative abnormalities in dense granules that impair their ability to accumulate normal contents rather than to an absence of dense granules per se.

Total mepacrine uptake in our studies into megakaryocytes, in contrast to platelets, was relatively low and also was not significantly different in normal and mutant mice. Thus the mutant dense-granule defects (ie, absence of dense granule contents) appear either very late in megakaryocyte development or early in platelet formation. The finding that a few (~1%) normal megakaryocytes flashed while no flashing was detectable in any mutant megakaryocytes suggests that only a small population of megakaryocytes, possibly a very late stage in maturation, contains mature dense granules. Since flashing was never seen in the mutant cells, the mutant defects likely are already present in megakaryocytes. The absence of flashing of dense granules of mutants illustrates that mutant dense granules express their immature phenotype when formed.

The low mepacrine accumulation in megakaryocytes is consistent with what is known of the timing of the accumulation of dense granule contents in normal platelets. For example, Mishory and Danon[42] found that newly formed platelets entering the bloodstream have practically no osmophilic organelles and that the number and size of these organelles increases with the age of the platelets.

The fact that mepacrine uptake is equal in normal and mutant megakaryocytes is consistent with the idea that the dense granule defect in all mutants is not in production of dense granules, since immature dense granules are present in mutant megakaryocytes. Rather, the defect seems to reside in the ability of dense granules to accumulate normal contents. Previous experiments utilizing bone marrow transplantation have shown that factors extrinsic to bone marrow precursor cells are not defective.[15,16] Also, initial rates of accumulation of one dense granule component, serotonin, are normal,[1,14] suggesting a normal transport mechanism. The defects in mutant platelets, therefore, are more likely to lie in mechanisms for retention of dense-granule components.

It is possible that some other substance(s) is present in mutant dense granules that prevents accumulation of dense-granule components and inhibits flashing. This is unlikely, at least in regard to primary gene products, since it is expected that a recessive mutation would result in a deficiency, rather than an increase, in the gene product. It remains possible that accumulation of such hypothetical substances could occur in mutant granules as a secondary effect of the mutations.

One uncertainty in interpretation of our megakaryocyte data is that, in comparison to platelets, there is less independent evidence that mepacrine uptake is principally into dense granules. The fact, however, that megakaryocytes incorporate labeled serotonin[43] is evidence that they are capable of concentrating normal dense-granule components. Also, Hourdille' et al[44] considered their finding of lowered mepacrine accumulation in megakaryocytes of a patient with a dense-granule defect as evidence that the mepacrine is incorporated mainly into dense granules.

Relatively few studies have been performed on dense granules of megakaryocytes of human storage pool disease. Hourdille' et al[44] found, in contrast to our results with mutant mice, that 74% of megakaryocytes of a patient with HPS showed no labeling with mepacrine and concluded, therefore, that the platelet anomaly is secondary to a megakaryocyte anomaly. Richards and Da Prada[35] found no positive uranaffin reaction in megakaryocytes of HPS patients or beige mice or fawn-hooded rats. However, this same method of detection also revealed[34] no positive reaction in platelets of the above mutant animals while we and Lorez et al[38] have readily detected dense granules in platelets of these mutants using the mepacrine-labeling technique. Therefore, as previously discussed, the mepacrine method may be a more sensitive method of detecting the relatively immature dense granules of megakaryocytes. Further studies on megakaryocytes of SPD patients are needed for an adequate comparison to our results using the various mouse mutants.

These results show that the normal accumulation of dense granule contents is under multigenic control with at least seven genes involved. The molecular bases for the organelle defects in the mouse mutants are still unknown. A speculative explanation of our results is that the mutants have defects in the membrane of dense granules. Probably such defects are also present in lysosomes and melanosomes,[8,9] emphasizing the relatedness of the three organelles. Our current focus is on identifying the primary gene products involved in the mouse models of SPD.

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The Presence of Clonogenic Cells in High-Grade Malignant Lymphoma: A Prognostic Factor

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Mark D. Minden, and Hans A. Messner

A culture system has been developed that promotes growth of clonogenic lymphoma cells of some patients with intermediate and high-grade malignant lymphoma. The formation of colonies in bone marrow, lymph nodes, and peripheral blood samples is best supported by human plasma. Colony formation of some patients was dependent upon growth factors, which in this study were added in the form of medium conditioned by phytohemagglutinin (PHA)-stimulated leukocytes (PHA-LCM). Some gave rise to lymphoma colonies without PHA-LCM but improved their frequency with PHA-LCM; others were completely independent of PHA-LCM. Colonies grown in primary cultures were routinely recloned and propagated as Epstein-Barr virus (EBV)-negative cell lines with stable B cell phenotype. The cell lines showed the same immunoglobulin rearrangement pattern as that observed in the primary lymphoma sample. In addition, a significant clinical correlation was observed between culture data and clinical outcome. Survival of patients who formed lymphoma colonies at any time during their clinical course was significantly shorter than survival of patients who did not give rise to colonies ($P = 0.0009$). The same observation was made when the survival assessment was performed for the subset of patients studied at diagnosis ($P = 0.0014$).

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THE MALIGNANT non-Hodgkin's lymphomas represent a diverse group of neoplasms with heterogeneous histopathologic[1,2] and clinical presentations.[3,4] A similar degree of variation is apparent when cellular properties are studied. Analysis of cell surface antigens with monoclonal antibodies (MoAbs) indicates that lymphoma cells of individual patients may reflect different stages of normal B cell or T cell differentiation.[5-7] Cell culture conditions are available to grow normal and abnormal B cells; a number of investigators have contributed to the evaluation of normal and abnormal B cell proliferation in cell culture.[8-13] Although the cell culture systems vary in detail, they all have in common the requirement for either feeder cells, cell-derived stimulating molecules, and/or mitogens such as *Staphylococcus aureus* protein A (SPA) or anti-immunoglobulin antibodies. These studies indicate that the proliferation and maturation of normal B cells may require a series of signals that are mediated by molecules[14-22] defined as B cell activator, B cell growth factor, and B cell maturation factor. It is not known whether clonogenic populations of B cell neoplasms are subject to similar controls or whether they proliferate autonomously. The exact requirements for growth are unknown. Smith et al[12] found that cells of some B cell malignancies could grow in the presence of fetal calf serum (FCS) and medium, while we observed that the growth of myeloma colonies required human plasma as well as PHA-LCM.[23] We have used the same culture system to analyze bone marrow, peripheral blood, and lymph node samples of patients with lymphoma to identify whether or not a clonogenic tumor stem cell population can be detected and propagated for further studies. In addition we examined the prognostic significance of colony growth.

MATERIALS AND METHODS

Patient Material

The patient material was contributed by collaborating physicians located at the Princess Margaret Hospital, the Wellesley Hospital, and the Sunnybrook Medical Centre, University of Toronto. As part of their diagnostic workup, bone marrow, peripheral blood, and lymph node specimens were obtained from consenting, unselected patients presenting to the above institutions with the provisional diagnosis of malignant lymphoma. In addition, samples became available from patients with malignant lymphoma in relapse. The histologic diagnosis and clinical status of the patients was unknown to the investigators until the growth pattern in culture was unequivocally determined by following cultures for at least 2 months.

Preparation of the Samples

Bone marrow and peripheral blood samples were aspirated into heparinized syringes and centrifuged in 60% Percoll to obtain a mononuclear cell suspension of density less than 1.077 g/mL. Surgically obtained lymph node sections were cut with scissors and passed through a fine wire mesh to prepare a single cell suspension.

Cell Culture Studies in Semisolid Medium

Freshly prepared cell suspensions were plated in methylcellulose as previously described.[23-25] Briefly, cells were cultured in Iscove's modified Dulbecco's minimum essential medium (IDMEM; Gibco, Grand Island, NY), 30% normal human plasma prepared under platelet-poor conditions,[24] 5×10^{-5} mol/L 2 mercaptoethanol (2ME), (Sigma, St. Louis), and 0.9% methylcellulose (Dow Chemical Co, Midland, MI). Each sample was grown with and without 10% of PHA-LCM (PHA,HA15; Wellcome Diagnostics, Dartford, England).[26] Cells were plated in duplicates, bone marrow cells at 2×10^5/mL, peripheral blood cells at 4×10^5/mL, and lymph node cells at 5 to 10×10^5/mL in 35-mm Petri dishes (LUX, Lab-Tek Division, Miles Laboratories, Naperville, IL). The cultures were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. The plates were frequently inspected for the emergence of colonies and scored on days 10 to 14. One of the plates of each experimental group was then supplemented with an additional 1-mL

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layer of the above described culture medium to allow long-term observation of the cultures at least until day 30.

The content of the second primary culture plate of each group was resuspended in IDMEM,[23] replated under identical culture conditions, and observed for 6 to 8 weeks. This recloning procedure was performed for primary cultures of all patients independent of whether or not lymphoma colonies were identified.

Studies in Liquid Suspension Culture

Primary samples that contained sufficient cells were also used to initiate liquid suspension cultures in IDMEM, 20% normal human plasma, 5×10^{-5} mol/L 2ME, and 10% PHA-LCM. This medium was completely exchanged at weekly intervals. Lymphoma colonies propagated in semisolid medium were also transferred into liquid suspension cultures and maintained as cell lines under the above described conditions.

Phenotypic Characterization of Lymphoma Cells Propagated in Long-term Cultures

Cells maintained in semisolid or liquid long-term cultures were carefully washed in IDMEM and examined on multiple occasions with a panel of MoAbs directed against B cell specific surface or cytoplasmic antigens. B1, B2, B4, and PCA1 were a generous gift of Dr Lee Nadler (Dana Farber Institute, Boston). Antibodies against the μ, δ, γ, κ, and λ chains were obtained from Tago Inc, Burlingame, CA. The panel also included MoAbs against the common ALL antigen (CALLA, J5 Coulter Immunology, Hialeah, FL), Il-2 receptor (TAC, Dr L. Nadler), the T cell subsets T3, T4, T6, and T8 (Ortho Diagnostic Systems, Don Mills, Ontario), and determinants associated with myeloid cells. T101 was kindly provided by Dr Ivor Royston (San Diego, CA). Staining was performed according to the technique described by Smith et al[27] and analyzed by immunofluorescence microscopy.

Preparation and Analysis of DNA

Cells from lymph nodes were washed twice in phosphate-buffered saline and resuspended in 10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 1 mmol/L EDTA (TNE) with 1% sodium dodecyl sulfate (SDS), and 200 μg/mL proteinase K incubated at 37°C over night and extracted sequentially with phenol: chloroform and then twice with chloroform. The DNA was precipitated with isopropanol and resuspended in 10 mmol/L Tris pH 7.5 1 mmol/L EDTA. Ten micrograms of DNA was digested with the restriction enzyme EcoR1, separated by agarose electrophoresis, and transferred to nitrocellulose.[28]

Probes

The presence of EBV was determined using a probe to BAM H1k region of EBV provided by Dr G. Miller, New Haven, CT.[29] The structure of the Ig heavy chain locus[30] was determined using a 3kb EcoR1, Hind III fragment containing heavy chain J region provided by Dr P. Leder, Boston. DNA fragments were labeled by nick translation with ^{32}P.[31]

Histologic Assessment

All lymph node specimens were classified according to Rappaport[1] and the working formulation.[2]

Statistical Analysis

The survival of patients who formed lymphoma colonies and patients who did not form lymphoma colonies was examined by Kaplan-Meier analysis[32] and compared by the method described by Mantel.[33] The two groups were tested for differences in clinical attributes using the chi-square[34] or Mann-Whitney u test.[34]

RESULTS

Clinical Data

Sixty-nine patients with non-Hodgkin's lymphoma with varying histologic phenotype and clinical stage were entered into the study. The available specimens included 31 lymph nodes, 33 bone marrow aspirates, six peripheral blood samples, one soft tissue tumor, and one mediastinal mass. Three patients were studied using material of more than one tissue source. Thirty-nine samples were obtained at diagnosis, 30 during relapse, 49 specimens were involved with lymphoma, 20 did not show any detectable lymphoma cells upon histologic and cytologic assessment. Table 1 presents a summary of the various samples from patients with non-Hodgkin's lymphoma. As can be seen, the development of EBV-negative lymphoma colonies was restricted to samples derived from patients with intermediate or high-grade malignant lymphoma.

Table 1. Histologic Phenotypes, Staging, Cell Source and Growth Pattern of All Patients Studied in Cell Culture

Histologic Classification	No. of Patients	Staging				Tissue				No. of Growers
		I	II	III	IV	LN	BM	PB	Other	
Small lymphocytic	5	0	0	0	5	3	2	0	0	1†
Follicular small cleaved cell	13	1	0	5	7	4	8	1	0	0
Follicular mixed	10	1	1	3	5	5	5	0	0	0
Follicular large cell	0	0	0	0	0	0	0	0	0	0
Diffuse small cleaved cell	6	0	1	2	3	1	4	1	0	0
Diffuse mixed	4	1	0	1	2	3	1	0	0	1
Diffuse large cell	22	1	3	4	14	9	11*	4*	1	9
Immunoblastic	6	0	3	0	3	4	1	0	1	4‡
Lymphoblastic	0	0	0	0	0	0	0	0	0	0
Small noncleaved cell	3	0	1	2	0	2	1	0	0	1
TOTAL	69	4	9	17	39	31	33	6	2	16

*Three grown from both sources.

†EBV positive.

‡One of the four cell lines is EBV positive.

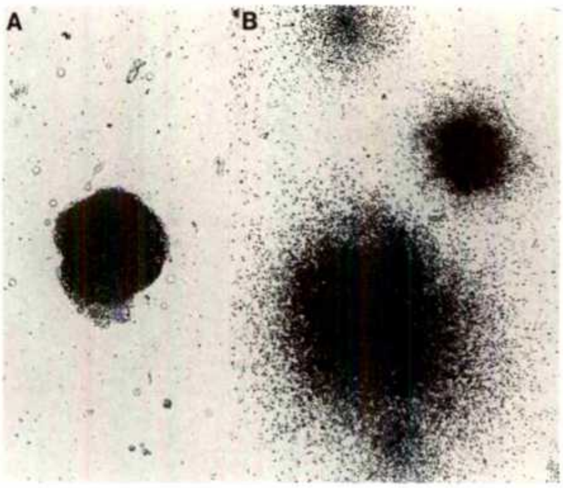

Fig 1. Variation in morphology of lymphoma colonies grown from different patients. Panel A displays a compact colony; panel B shows more loosely arranged colonies.

Lymphoma Colonies

Lymphoma colonies were grown from lymph nodes, bone marrow aspirates, and peripheral blood samples of 16 patients. The colonies were uniformly composed of round mononuclear cells and showed patient-related heterogeneity with respect to colony morphology, size, and plating efficiency. The majority of samples gave rise to compact colonies that contained between 500 and 10,000 cells (Fig 1A). Some specimens produced more loosely arranged colonies (Fig 1B). Usually colonies developed within seven to 20 days. Some cultures had to be followed for as long as 30 days to detect the formation of lymphoma colonies. The plating efficiency in primary cultures varied from 0.001 to 0.2% (Table 2). The primary plating efficiency could not be assessed for all cultures because of confluent colony growth.

While colonies grown from lymph node specimens were easily scorable as the only identifiable colony type, lymphoma colonies from bone marrow and peripheral blood samples usually developed among normal hemopoietic colonies. Compact lymphoma colonies could easily be distinguished. Identification of loosely arranged lymphoma colonies among normal hemopoietic colonies was more difficult and required confirmation by marker analysis.

Recloning Studies

Each of the primary cultures, independent of whether or not primary lymphoma colonies were observed, were resuspended in fresh medium and recultured under identical conditions. All samples that yielded primary lymphoma colonies gave rise to secondary lymphoma colonies. Growth in secondary cultures was usually associated with an increase in the plating efficiency (Table 2). Every one of the samples forming primary colonies could be propagated in semisolid medium for many generations using identical recloning procedures. None of the samples that did not form primary lymphoma colonies showed lymphoma colony growth upon replating.

Proliferation in Liquid Suspension Culture

Cells derived from these cultures could be adapted to liquid suspension culture conditions and maintained as cell lines. They are denoted as OCI-Ly 1 to 16 (Table 2). At time of last follow-up the lines had been maintained in culture for 9 to 35 months. Cultures of one patient (OCI-Ly16) were lost after 2 months due to fungal contamination. The details of the established cell lines, their plating efficiency, and

Table 2. Growth Characteristics of the Generated Lymphoma Cell Lines

			Plating Efficiency (%)			Doubling Time		
Cell Line	Histotype	Source	1	2	Late	Time (Hr)	Culture (Mo)	EBV Status
OCI-LY1*	Diffuse large cell	BM	0.002	3.9	24	24	35	–
OCI-LY2*	Diffuse large cell	BM + PB	0.002	0.005	4	40	35	–
OCI-LY3*	Diffuse large cell	BM + PB	0.005	3.3	16	24	33	–
OCI-LY4	Immunoblastic	LN	0.001	—	3	17	26	–
OCI-LY5	Small lymphocytic	LN	NA†	NA†	2	21	22	+
OCI-LY6	Diffuse large cell	LN	Con‡	Con‡	2	48	22	–
OCI-LY7*	Diffuse large cell	BM + PB	Con	Con	1	15	20	–
OCI-LY8	Immunoblastic	LN	0.024	—	4	24	20	–
OCI-LY9	Diffuse large cell	BM	NA	NA	12	48	18	–
OCI-LY10	Immunoblastic	LN	0.20	—	3	39	14	–
OCI-LY11	Diffuse large cell	LN	0.02	0.05	14	29	14	–
OCI-LY12	Small noncleaved cell	LN	0.02	0.31	0.1	>100	12	–
OCI-LY13.1	Diffuse large cell	BM	0.01	0.55	2	29	11	–
OCI-LY13.2*		BM	0.05	4.0	6	20	7	–
OCI-LY14	Diffuse mixed	LN	NA	NA	6	86	11	–
OCI-LY15	Immunoblastic	BM	0.01	0.02	3	53	9	+
OCI-LY16	Diffuse large cell	LN	Con	Con	0.2	ND†	2§	–

*Lines established from samples obtained during relapse; the other lines were grown from specimens taken at diagnosis.

†Not assessable, primary growth in liquid.

‡Confluent growth at concentration plated.

§Lost to fungus.

doubling time are listed in Table 2. Their current plating efficiencies vary from 0.1% to 24%, with doubling times ranging from 15 to more than 100 hours.

Marker Studies

Cells propagated in semisolid and liquid suspension cultures were examined with a panel of MoAbs directed against surface antigens of various B, T, and myeloid cells. The data are presented in Table 3. Cells of all lymphoma lines expressed some B cell markers. They were observed in heterogeneous combinations without favoring a specific phenotype. Twelve of the lines were positive for B1, two for B2, ten for B4, and four for PCA1. Seven demonstrated cytoplasmic and 13 expressed cell surface immunoglobulins. In addition, seven reacted with anti-CALLA, and four stained with anti-TAC. A subpopulation of cells of three lines showed positive results with pan-T reagents, and one additional patient displayed T101 activity.

Lymphoma growth thus was not restricted to a specific marker phenotype. Repeat assessment of the cells using the same panel of antibodies demonstrated that the phenotype of all cell lines had not changed with propagation in culture.

Two cell lines were established from the same patient with the histopathologic diagnosis of diffuse large cell lymphoma, one at diagnosis (OCI-Ly 13.1) and one at relapse (OCI-Ly 13.2). Cells of the first line reacted with the MoAb against the IL 2 receptor; cells of the second line were negative.

Marker studies were performed on one of the primary tumor tissues (OCI-Ly8). These were in agreement with the markers observed on cells of the cell line after culture.

Immunoglobulin Rearrangement

The structure of the immunoglobulin genes in the primary lymph node sample and resulting cell line were determined in six cases. The rearrangement pattern observed in the lymph node samples was in every case identical to that of the resulting cell line. A representative example is depicted in Fig 2.

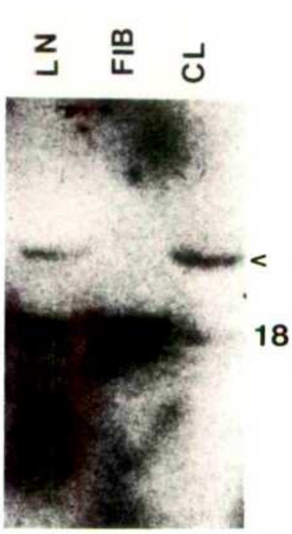

Fig 2. Immunoglobulin gene rearrangement pattern of lymph node cells (LN), skin fibroblasts (FIB), and lymphoma cells of the cell line (CL) OCI-Ly4, all derived from the same patient. The same rearrangement pattern was observed for the primary lymph node and the resulting cell line; the germ line band (18kb) is observed in DNA obtained from fibroblast cultures.

Growth Requirements

The contribution of various culture components to the growth of lymphoma colonies was assessed.

Influence of human plasma. All cell lines were examined to study the influence of human plasma in comparison to human serum derived from the same source and fetal calf serum. Plasma samples prepared under platelet-poor conditions supported linear growth even when very low numbers of cells were plated (Fig 3). In contrast, lymphoma lines grew suboptimally or not at all when platelet-poor plasma was replaced by human serum or fetal calf serum. However, cells of some lines propagated in liquid culture over long periods were adapted to fetal calf serum.

Role of PHA-LCM. The requirements of all cell lines for PHA-LCM were studied by performing dose-response curve analyses. Three growth patterns were identified, and representative examples are displayed in Fig 4. The growth of EBV-negative lymphoma colonies in nine of 14 examples were independent of added PHA-LCM. Addition of PHA-

Table 3. Marker Pattern of the Lymphoma Lines

Cell Line	B1	B2	B4	J5	PCAI	Surface					Cytoplasmic					Pan-T	T101	Tac
						μ	δ	γ	κ	λ	γ	δ	γ	κ	λ			
OCI-Ly1	+	–	+	+	–	+	–	–	+	–	+	–	–	+	–	–	–	–
OCI-Ly2	+	–	+	–	ND	–	–	–	–	–	+	–	–	+	–	–	–	–
OCI-Ly3	+	–	+	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–
OCI-Ly4	+	+	+	–	–	+	–	–	+	–	–	–	–		–	–	–	–
OCI-Ly5	+	ND	ND	–	ND	–	–	–	–	+	–	–	–	–	+	ND	ND	ND
OCI-Ly6	+	–	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–
OCI-Ly7	+	–	+	+	–	+	–	–	+	–	+	–	–	+	–	–	–	–
OCI-Ly8	+	–	+	+	ND	+	+	–	–	+	+	–	–	–	+	–	–	–
OCI-Ly9	+	–	+	–	ND	–	–	+	–	–	+	–	–	+	–	–	+	–
OCI-Ly10	+	–	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–
OCI-Ly11	+	–	+	+	–	+	–	–	+	–	ND	ND	ND	ND	ND	–	–	–
OCI-Ly12	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	+	+	+
OCI-Ly13.1	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	+
OCI-Ly13.2	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	+
OCI-Ly14	–	–	–	–	+	–	–	–	–	–	ND	ND	ND	ND	ND	–	–	+
OCI-Ly15	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	+
OCI-LY16 lost prior to analysis																		

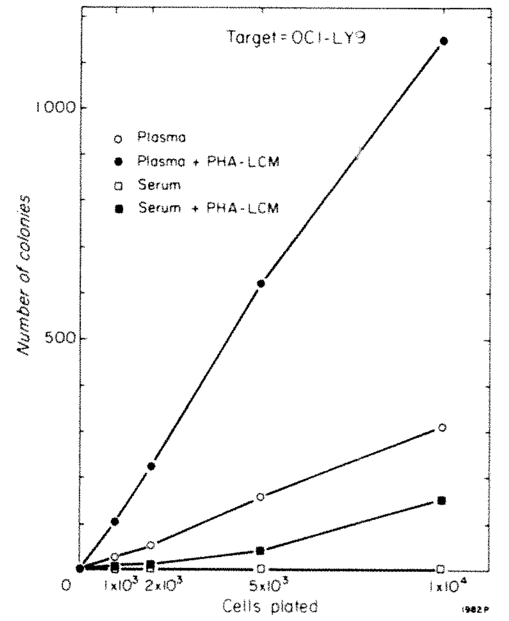

Fig 3. Influence of human plasma, serum, and PHA-LCM on the growth of lymphoma colonies.

LCM did not alter the frequency, size, or reclonability of the colonies. Four of 14 patients demonstrated formation of some colonies in the absence of PHA-LCM. Addition of PHA-LCM to these enhanced the number of colonies without influencing their size. Two cell lines (OCI-Ly 9 and OCI-Ly 13.1) gave rise to only a few small clusters of cells in the absence of PHA-LCM. Both lines formed numerous large colonies in the presence of PHA-LCM. One patient was studied on two separate occasions. Bone marrow samples became available at diagnosis (OCI-Ly 13.1) and four months later during relapse (OCI-Ly 13.2). Colony formation in the first sample was dependent upon PHA-LCM, yielding a plating efficiency of 70 colonies per 2×10^4 mononuclear cells. The second sample gave rise to 675 colonies per 2×10^4 mononuclear cells in the absence of PHA-LCM. The plating efficiency of the second sample increased to 1,240 per 2×10^4 mononuclear cells when PHA-LCM was added. The data are displayed in form of a linearity study in Fig 5.

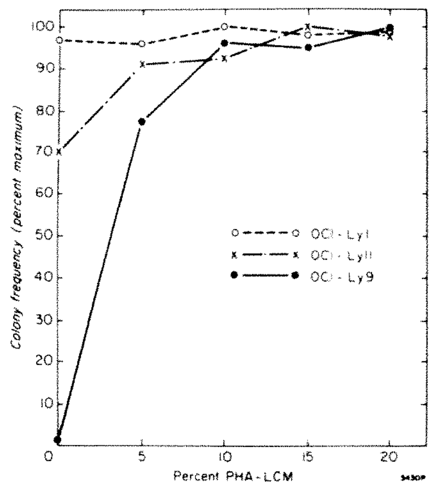

Fig 4. PHA-LCM dose-response curves representing three different patterns. Each curve was established using cells of a representative cell line.

Clinical Correlations

The examined patients represented a heterogeneous population with respect to histologic phenotype, histologic involvement of the cultured samples with lymphoma, timing of culture with respect to diagnosis, and growth pattern in culture.

The formation of EBV-negative lymphoma colonies was only observed in specimens derived from patients with lymphoma of intermediate and high-grade malignancy. The evaluation of the prognostic value of colony formation was therefore restricted to this subset of 35 patients. In addition, the patient who gave rise to the EBV-positive line OCI-Ly 15 was eliminated from the clinical analysis.

Twenty-five of the remaining 34 patients were studied at diagnosis, ten were cultured during relapse. Twenty-seven of the 34 specimens were histologically involved with lymphoma. Seven did not show signs of tumor involvement; all of the latter were bone marrow specimens. The samples of 14 patients gave rise to EBV-negative lymphoma colonies. Nine were obtained at diagnosis; five during relapse (Table 4). Specimens of 13 patients did not grow. Ten of these were collected at diagnosis; three during relapse. Both groups of patients were evenly matched for sex, histologic diagnosis, staging, and presence of B symptoms (all P values > 0.1 by chi-square analysis). The median age of nongrowers was higher than that of growers ($P = 0.009$, Mann-Whitney).

The majority of patients received BACOP combination

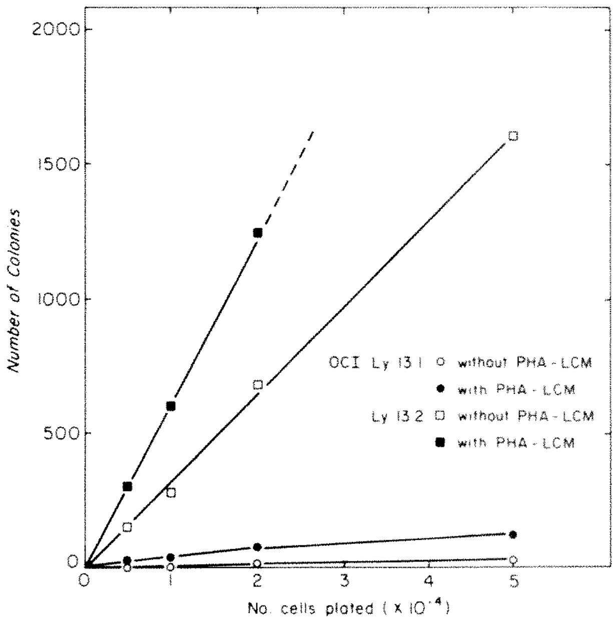

Fig 5. Cloning efficiency with and without PHA-LCM of lymphoma colony-forming cells obtained at diagnosis (OCI Ly 13.1) and at relapse (OCI Ly 13.2) of the same patient.

Table 4. Characterization of the Studied Patients With Intermediate and High-Grade Malignant Lymphoma

Attributes		Growers	Nongrowers
Number		14	20
Age (years, median, and range)		50 (21-73)	66 (26-86)
Sex M		10	11
F		4	2
Source of tissue	LN	8	9
	BM	4*	9
	PB	3*	0
	Other	0	2
Histology	Diffuse mixed	1	3
	Diffuse large cell	9	13
	Immunoblastic	3	2
	Small noncleaved cell	1	2
Stage I		0	2
II		3	4
III		2	5
IV		9	9
B symptoms		9	9
BM involvement		9	8
Number surviving		1	13
Causes of death	Sepsis	2	1
	Progressive disease and sepsis	10	6
	Other†	1	—

*One sample grown from both sources.

†Patient died with carcinoma of the lung.

chemotherapy (bleomycin, adriamycin, cyclophosphamide, oncovin, and prednisolone)[35]; one was treated with PRO-MACE-MOPP (epipodophyllotoxin, cyclophosphamide, adriamycin, methotrexate, and prednisone).[36] Patients were observed for 6 to 35 months with a median of 14 months. The time of follow-up is the same for both subgroups of patients. Eight of the 13 patients who did not form lymphoma colonies are currently alive. One patient died with sepsis during inductive chemotherapy; four patients died with progressive disease.

In contrast, only one patient is surviving of the 14 who formed lymphoma colonies. Two died with septicemia, ten with progressive disease unresponsive to further therapy, and one patient died with a coexisting carcinoma of the lung. The cell line OCI-Ly 12 derived from the lone survivor is characterized by a doubling time that exceeds 100 hours and is the slowest of all lymphoma lines.

The survival of both groups was evaluated and displayed as Kaplan-Meier plots.[32] The curves were compared according to Mantel.[33] The analysis was performed for the whole group of 34 patients and for selected subgroups as listed in Table 5.

The median survival from diagnosis of all patients who formed lymphoma colonies was 7 months. In contrast, patients who did not form lymphoma colonies showed a significantly longer median survival of 25 months ($P = 0.0009$, Table 5, Fig 6).

A similar observation was made when only patients who were studied with cell cultures at diagnosis were included in the analysis. The median survival of growers was 6 months, while the median for nongrowers was not reached at 22 months. A comparison of the survival curves yielded a significant difference with a P value of 0.0014 (Table 5, Fig 6).

The median survival of growers was also significantly shorter if only the 27 patients who demonstrated histologically involvement with lymphoma of the plated specimen were analyzed (Table 5).

Nine patients were examined in relapse. Five of these grew lymphoma colonies. All five patients died within 2 months after relapse. Their median survival from diagnosis was 13 months. Two of the four patients who did not form lymphoma colonies died within 2 months, one patient survived 3 months, and one is alive 7 months after the study. The median survival from diagnosis for this subgroup was 18 months. These groups are too small to allow any meaningful statistical comparison.

DISCUSSION

A number of risk factors affecting prognosis can be identified in patients with lymphoma. These include histologic phenotypes, stage of disease, cytogenetic parameters,[37,38] expression of the transferrin receptor,[39] and response to chemotherapy.[35,36] Particularly, the latter segregates patients in the high-risk group who may have a long-term therapeutic benefit from patients who will succumb early to progressive disease. Recent encouraging results in autolo-

Table 5. Comparison of Survival of Patients With Intermediate and High-Grade Malignant Lymphoma Who Form or Do Not Form Lymphoma Colonies in Culture

Patient Groups	Number	Survival From Date Of	Growers (n)	Median Survival (mo)	Nongrowers (n)	Median Survival (mo)	Comparison of Survival Curves P value (Savage)
1*	34	Diagnosis	14	7	20	25	0.0009
	34	Culture	14	2	20	21	0.0005
2†	27	Diagnosis	14	7	13	21	0.0287
	27	Culture	14	2	13	21	0.0026
3‡	25	Diagnosis	9	6	16	Not reached at 22	0.0014
4§	20	Diagnosis	9	6	11	21	0.0053

*1 Includes all patients.

†2 Includes only patients whose cultured tissues were histologically involved by lymphoma.

‡3 Includes only patients with culture results obtained at diagnosis.

§4 Includes only patients with culture results obtained at diagnosis and tumor involvement of the cultured specimen.

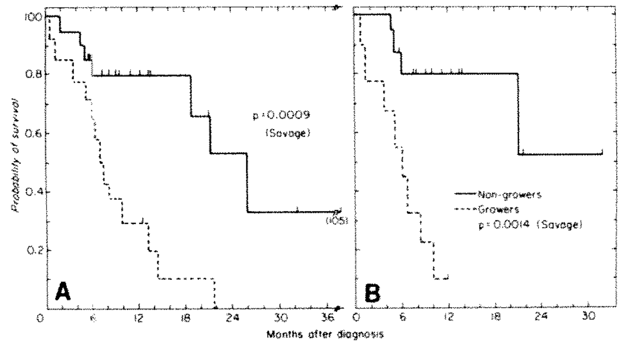

Fig 6. Kaplan-Meier survival curves of patients with intermediate and high-grade malignant lymphoma who formed lymphoma colonies and EBV-negative cell lines in comparison to patients who did not form colonies in culture. Panel A represents survival of patients (n = 34) studied either at diagnosis or relapse. Panel B depicts the survival curves for patients examined at diagnosis. Comparison of the survival curves in both panels yielded a significant difference using the generalized Savage test.

gous and allogeneic bone marrow transplantation of patients with lymphomas have suggested that some patients who have relapsed with progressive disease may still be salvageable by transplantation.[40-43] This therapeutic option may be used with greater advantage earlier in the clinical course.

A culture system is described that promotes growth of lymphoma colonies in bone marrow, peripheral blood, and lymph node samples of a subgroup of patients with lymphoma belonging to a high-risk group based on histology. Growth was observed at the initial assessment or at times of re-examination for disease recurrence.

A number of observations strongly support the view that the clonogenic cells detected in the system are part of the original disease. First, whenever examined the cell lines demonstrate the same clonal immunoglobulin gene rearrangement pattern as that observed in the primary tumor. Second, the same immunoglobulins were observed on cells of the cell lines and the primary lymphoma tissue. Third, cells of the cell lines are EBV-negative and can be propagated indefinitely in liquid suspension or semisolid culture. Fourth, patients whose lymphoma cells grew in culture had a significantly poorer prognosis.

The system described here is therefore useful in identifying patients at the time of diagnosis as potential treatment failures. This group of patients may be considered earlier in their clinical course for innovative treatment approaches, such as bone marrow transplantation.

In addition to being useful as a prognostic parameter, the cell culture system may provide insight into biological aspects of malignant lymphoma. One of the areas to be explored is the heterogeneous requirement of clonogenic lymphoma cells for PHA-LCM. Clonogenic lymphoma cells of some patients are dependent upon addition of PHA-LCM to the culture. This suggests that these cells require exogenous signals for their proliferation. Some cell lines give rise to a few lymphoma colonies without PHA-LCM but show an increased plating efficiency in the presence of PHA-LCM. The third group of patients forms lymphoma colonies without PHA-LCM, and improves neither their frequency nor their size in the presence of PHA-LCM. These data may either suggest the existence of clonogenic subpopulations with differing requirements for PHA-LCM or may indicate the autocrine or paracrine production of PHA-LCM-like stimulatory substances by lymphoma cells. The availability of cell lines that have retained the various types of PHA-LCM requirement will facilitate studies to delineate the underlying mechanism. They will also provide the opportunity to compare the activity in PHA-LCM with defined purified or recombinant hemopoietic growth factors.

Observations on a single patient have provided a further lead to understand the heterogeneity in PHA-LCM requirement. This patient was initially studied at diagnosis. Colony formation of a bone marrow sample was almost completely dependent upon PHA-LCM. The patient went into a complete remission and was re-examined four months later during relapse. The growth requirement of clonogenic lymphoma cells obtained from a bone marrow specimen at that time had changed. A large number of colonies were observed without PHA-LCM. The colony frequency and size increased only slightly in its presence. The patient did not respond to further therapy and died with progressive disease shortly after the time of study. These results suggest that the requirement for PHA-LCM may change during the clinical course of an individual patient. In this isolated example a decrease in PHA-LCM requirement reflected disease progression. If verified by kinetic studies in other patients the PHA-LCM requirement may, in addition to colony growth, provide a further prognostic indicator.

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The Pathogenesis of Accelerated Fibrinolysis in Liver Cirrhosis: A Critical Role for Tissue Plasminogen Activator Inhibitor

By Steven L. Hersch, Thomas Kunelis, and Robert B. Francis, Jr

The pathogenesis of accelerated fibrinolysis in liver cirrhosis was investigated by comparing the results of specific assays for tissue plasminogen activator (tpa) antigen, tpa activity, tpa inhibitor, and alpha-2 plasmin inhibitor (a2PI) in 12 patients with cirrhosis and markedly accelerated fibrinolysis (dilute whole blood clot lysis time (DWBCLT) < two hours), in nine patients with cirrhosis and moderately accelerated fibrinolysis (DWBCLT two to four hours), and in nine patients with cirrhosis and normal fibrinolysis (DWBCLT > four hours). Mean tpa antigen was markedly increased in all three groups, but no correlation was observed between overall fibrinolytic activity as measured by the DWBCLT and the level of tpa antigen. In contrast, there was a significant correlation between overall fibrinolytic activity and tpa activity and an equally significant correlation between fibrinolytic activity and decreased tpa inhibition. Mean a2PI activity was significantly lower than normal in groups 1 and 2 but was normal in group 3. The pathogenesis of accelerated fibrinolysis in liver cirrhosis thus appears to depend critically on the capacity of plasma inhibitors to inhibit increased circulating tpa antigen. Reduced a2PI also appears to play a role.

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ACCELERATED fibrinolysis is a recognized complication of cirrhosis.[1-3] The liver is not only an important site of clearance of circulating plasminogen activators[4] but also synthesizes the main plasmin inhibitor, alpha-2 plasmin inhibitor (a2PI).[5] Thus a multifactorial pathogenesis of accelerated fibrinolysis in cirrhosis has been presumed, with contributions from elevated levels of circulating plasminogen activators caused by decreased clearance[6,7] and reduced inhibition of plasmin due to diminished a2PI synthesis.[8] Previously published studies of fibrinolysis in cirrhosis have not, however, investigated the interrelationship of multiple abnormalities of the fibrinolytic system, nor have they attempted to correlate these abnormalities with either overall fibrinolytic activity of blood or with clinical manifestations. Moreover, the recent discovery of one or more rapid inhibitors of tissue-type plasminogen activator (tpa) in plasma[9-11] has revealed a new complexity in the physiologic regulation of fibrinolysis. The behavior of tpa inhibitor in liver cirrhosis has yet to be investigated.

We previously reported that accelerated fibrinolysis may have significant clinical consequences for individuals with liver cirrhosis.[12] In comparing bleeding histories of 46 patients with markedly accelerated fibrinolysis (DWBCLT < two hours) to 44 patients with normal fibrinolysis (DWBCLT > four hours), we found that patients with accelerated fibrinolysis were at increased risk for major soft-tissue hemorrhage after trauma and exhibited a trend toward increased intracranial bleeding, usually fatal.[12] Other hemostatic abnormalities may have contributed to the increased bleeding in patients with accelerated fibrinolysis, however, since this group had a significantly longer mean partial thromboplastin time (PTT) and higher mean levels of fibrin/fibrinogen degradation products (fdp/FDP) than the group with normal fibrinolysis. Nevertheless, in several patients with severe bleeding, accelerated fibrinolysis was the only marked hemostatic abnormality present.

This study raised many important questions. What specific abnormalities of fibrinolysis contribute to accelerated fibrinolysis in patients with cirrhosis? Which are the most important? Why does fibrinolytic activity vary so greatly among individuals with apparently equally severe liver disease? In an attempt to answer these questions, we undertook a comprehensive evaluation of the fibrinolytic system in patients with liver cirrhosis using newly developed, highly specific assays for tpa antigen and activity, tpa inhibitor, and a2PI.

MATERIALS AND METHODS

Patient selection. The records of the Coagulation Laboratory of the Division of Hematology at Los Angeles County (LAC) University of Southern California (USC) Medical Center were reviewed to identify those patients with liver disease in whom fibrinolytic activity had been measured with the DWBCLT. The diagnosis of cirrhosis was confirmed by a review of the medical records for each patient identified. Patients were considered to have cirrhosis if they showed either biopsy evidence of cirrhosis or documented portal hypertension (presence of esophageal varices or redistribution of tracer to spleen and bone marrow on liver-spleen scanning) in the setting of parenchymal liver disease and appropriate historic background (heavy alcohol consumption or chronic hepatitis). Patients with acute hepatic necrosis secondary to viral or toxic agents were excluded. All patients meeting these criteria were included in the analysis, provided sufficient stored plasma obtained at the same time as the DWBCLT was available for performance of all the fibrinolytic assays. To determine the possible influence of transfused blood products on the results of fibrinolytic assays, the records of the LAC-USC Medical Center blood bank were reviewed, and notation was made of the quantity and type of all blood products received in the seven days preceding the date of plasma sampling. In calculating the total amount of plasma received by each patient, we assumed that one unit of fresh frozen plasma contained 225 mL of plasma, one unit of packed red cells 75 mL, and one unit of platelets 50 mL. For determination of the normal range for fibrinolytic assays, blood

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was obtained from resting healthy adult donors without history of bleeding or thrombosis. The principles of the Declaration of Helsinki were followed in the care of all patients.

Preparation of plasma samples and euglobulin precipitates. All samples were obtained by atraumatic venipuncture and anticoagulated with one-tenth volume of balanced citrate. Plasma was prepared by centrifugation for ten minutes at 10,000 rpm at 4°C and stored at −20°C or −85°C until assayed. The euglobulin fraction of plasma was obtained by diluting 0.5 mL of plasma in 20 volumes of ice-cold 0.014% acetic acid (pH 5.6) prepared fresh daily from a 1.4% stock solution. After incubating for at least 30 minutes on melting ice, the precipitate was recovered by centrifugation for ten minutes at 2,500 rpm at 4°C and resuspended in 0.5 mL of 0.01 mol/L phosphate, 0.15 mol/L NaCl, pH 7.4, containing 0.1% (vol/vol) Tween 80 (PBS-Tween).

Materials. Tissue plasminogen activator (tpa) was purified from the conditioned medium of Bowes human melanoma cell cultures as previously described.[13] Human plasminogen (glu/lys mixture) and goat antihuman tpa antibody were obtained from American Diagnostica (Greenwich, CT). Cyanogen bromide-fragmented fibrinogen was prepared as previously described.[14] D-valleu-lys-pNA (S-2251) was obtained from Helena Laboratories (Beaumont, TX). Crystallized bovine serum albumin was obtained from Sigma Chemical (St. Louis). All other materials were of the highest quality obtainable.

Measurement of tpa antigen. The ELISA method of Bergsdorf et al,[15] commercially available through American Diagnostica (Greenwich, CT), was used. Briefly, 96-well flat-bottomed microtiter plates (Nunc Immunoplate I-F) were coated overnight at room temperature with goat antihuman tpa antibody, followed by washing with 0.15 mol/L NaCl containing 0.1% (vol/vol) Tween 80 (saline-Tween), and blocking of unreacted binding sites with 2% bovine serum albumin (BSA) for one to two hours at room temperature. After washing again, diluted euglobulin precipitates of test plasmas and a range of concentrations (0.05 to 1.6 ng/mL final in the wells) of purified tpa standard (as supplied by the manufacturer) were added and allowed to incubate overnight at room temperature. After additional washing, goat antihuman tpa antibody conjugated to peroxidase was added and allowed to incubate for two hours at room temperature. Then the plate was washed again, and ortho-phenylene diamine substrate was added. After 30 minutes in the dark at room temperature, the reaction was stopped by adding 4.5 mol/L H_2SO_4, and the absorbance at 492 nm was read on an eight-channel scanning spectrophotometer (Titertek Multiskan, Flow Laboratories, Irvine, CA). Each euglobulin precipitate was assayed in duplicate at four dilutions in PBS-Tween (fourfold, eightfold, 16-fold, and 32-fold), and the mean value of all dilutions falling on the linear portion of the standard curve was reported. Tpa antigen values in 25 healthy subjects were normally distributed, with a mean of 4.9 plus or minus 2.6 ng/mL (SD) and a range of 0.9 to 11.2 ng/mL. With this assay, quantitative recovery of a wide range of concentrations of purified tpa added to plasma (5 to 50 ng/mL final) was observed, indicating that both free tpa and the tpa/tpa inhibitor complex were detected with equal efficacy.

Measurement of tpa activity. A solid-phase bioimmunoassay similar to that described by Mahmoud and Gaffney[16] was employed to measure tpa activity in euglobulin precipitates of plasma. Microtiter plates were coated overnight with goat antihuman tpa antibody followed by washing and blocking with 2% BSA as described above. Then euglobulin precipitates and a range of concentrations of purified tpa standard (as supplied for the tpa ELISA) were added and incubated for one to two hours at room temperature. After washing, purified human plasminogen (10 μg/mL final) and the plasmin substrate S-2251 (0.15 mmol/L final) in PBS-Tween were added, and color was allowed to develop overnight at 37°C. Absorbance was read at 405 nm on the eight-channel spectrophotometer. Each euglobulin precipitate was assayed in duplicate at three dilutions (twofold, fourfold, and eightfold) and the mean value reported. With this assay tpa activity was not detectable in plasma from most resting normal subjects.

Measurement of tpa inhibitor. The assay of Juhan-Vague et al[17] was used. Tissue plasminogen activator (tpa) purified from Bowes human melanoma cell cultures was added to prewarmed plasma at a final concentration of 50 ng/mL and allowed to incubate for exactly five minutes at 37°C, followed by acidification and recovery of the euglobulin precipitate as described above. The residual tpa activity was measured in microtiter plate wells, as described by Verheijen et al,[14] using cyanogen bromide-fragmented fibrinogen as a stimulant. Since with this method the presence of free tpa activity in the test plasmas could result in a falsely low apparent tpa inhibitor level, each test plasma was also assayed without addition of purified tpa, and the difference in activity between the samples with and without added tpa was used to calculate the tpa inhibitor level. Tissue plasminogen activator (tpa) inhibitor was expressed as ng/mL of added tpa inhibited. Mean tpa inhibitor in 19 normal healthy subjects was 41.9 plus or minus 9.8 ng/mL.

Measurement of a2PI activity. The a2PI activity in plasma was measured spectrofluorometrically using the commercial "Protopath" assay of Dade (Miami), modified according to the method of Matsuda et al[18] to eliminate any effect of alpha-2 macroglobulin on the assay. Briefly, plasma samples were incubated for five minutes at 37°C with Tris-saline buffer pH 7.5 containing 0.2 mol/L methylamine HCl to inhibit alpha-2 macroglobulin, followed by addition of plasmin, further incubation for exactly one minute at 37°C, and measurement of residual plasmin activity spectrofluorometrically. Results of test samples were expressed as a percentage of the activity in pooled normal plasma. The normal range for 15 healthy subjects was 73% to 119% (mean ±2 SD).

Other assays. The DWBCLT was performed as previously described.[12] Briefly, 0.2 mL of freshly drawn blood was pipetted into 1.7 mL of ice-cold acetate-citrate buffer pH 7.4, inverted several times to mix, and kept on melting ice until 0.1 mL of a 50 U/mL thrombin solution could be added. Duplicate samples so processed were incubated at 37°C until lysis occurred. Quick prothrombin times and activated partial thromboplastin times were performed in standard fashion. Levels of fdp/FDP were determined using the Thrombo-Wellcotest (Burroughs-Wellcome, Research Triangle Park, NC).

Statistical methods. Significance of differences in mean laboratory parameters among the patient groups and normal subjects was assessed using the two-tailed Student's *t* test, with a P value of <0.05 considered to be significant. Correlation coefficients were calculated using simple linear regression.

RESULTS

Thirty patients, accrued between January 1983 and June 1986, had sufficient plasma available for performance of all fibrinolytic assays. Twelve patients (group 1) had markedly accelerated fibrinolysis (DWBCLT < two hours). Nine patients (group 2) had moderately accelerated fibrinolysis (DWBCLT between two and four hours). Nine patients (group 3) had normal fibrinolysis (DWBCLT > four hours). Results of standard coagulation assays and liver function tests are displayed in Table 1. Although group 1 patients had a lower mean serum albumin, higher mean total bilirubin, longer mean prothrombin time and PTT, and higher level of fdp/FDPs than groups 2 and 3, the only statistically significant difference among the three groups was observed for the

Table 1. Coagulation and Liver Function Tests (Mean ± SD)

	Group 1	Group 2	Group 3
Serum albumin (g/dL)	2.6 ± 0.5	2.7 ± 0.6	3.0 ± 0.6
Total bilirubin (mg/dL)	8.3 ± 6.4	4.4 ± 2.6	4.1 ± 4.7
Fibrinogen (mg/dL)	119 ± 51	214 ± 43	276 ± 118
Prothrombin time (seconds)	22.7 ± 5.2	18.7 ± 3.3	19.3 ± 5.4
PTT (seconds)	102.8 ± 49.9	74.9 ± 15.1	74.9 ± 41.3
fdp/FDP (μg/mL)	22.0 ± 28.5	17.8 ± 25.7	17.8 ± 16.8
Plasma received (mL)	480 ± 690	344 ± 601	1661 ± 2937

fibrinogen level, which was significantly lower in groups 1 and 2 than in group 3.

Fibrinolytic data are summarized in Table 2. A similarly marked increase in mean tpa antigen was seen in all three groups when compared with the values obtained in normal subjects. Tpa antigen showed no correlation with overall fibrinolytic activity as measured by the DWBCLT ($r = -0.13$, $P > 0.20$). In contrast, tpa activity was significantly correlated with the DWBCLT ($r = -0.45$, $P < 0.05$) and differed markedly among the three groups, being markedly increased in group 1 and essentially undetectable in group 3. The tpa inhibitor also correlated significantly with the DWBCLT ($r = 0.43$, $P < 0.05$) and was significantly lower in group 1 than in group 3. The tpa inhibitor did not correlate significantly with serum albumin ($r = -0.09$, $P > 0.50$) but did correlate significantly with a2PI ($r = 0.47$, $P < 0.05$).

Mean a2PI activity was significantly lower than normal in groups 1 and 2 and was normal in group 3. The a2PI did not correlate significantly with the DWBCLT ($r = 0.35$, $P > 0.05$) or with serum albumin ($r = 0.32$, $P > 0.10$) but did correlate significantly with fibrinogen ($r = 0.59$, $P < 0.01$) and with tpa inhibitor ($r = 0.47$, $P < 0.05$).

Five patients in group 1, three patients in group 2, and five patients in group 3 received blood products prior to measurement of fibrinolytic parameters. The amount of plasma received did not differ significantly among the three groups and did not correlate significantly with any of the fibrinolytic parameters.

DISCUSSION

Tissue plasminogen activator (tpa) is synthesized by endothelium and cleared by the liver. Thus diminished hepatic clearance in cirrhosis would be expected to produce elevated levels of circulating tpa. Using the ELISA technique developed by Bergsdorf et al,[15] we found that marked increases in tpa antigen are common in patients with cirrhosis. Surprisingly, however, the level of tpa antigen did not correlate with overall fibrinolytic activity as measured by the DWBCLT. In contrast, the level of free tpa activity correlated strongly with overall fibrinolytic activity. We interpret this to indicate that in patients with cirrhosis the amount of tpa inhibitor available to bind and neutralize circulating tpa is a critical factor in the development of accelerated fibrinolysis. Those patients whose inhibitory capacity is adequate show no increased fibrinolytic activity, even if tpa antigen is markedly elevated. Patients with insufficient inhibitor exhibit increased fibrinolytic activity proportional to the amount of tpa activity that escapes inhibition. This interpretation is supported by the finding that tpa inhibitor activity was lowest in group 1 and highest in group 3 and correlated significantly with the DWBCLT.

Total tpa antigen in plasma is the sum of free tpa and tpa in complex with its inhibitors.[19-21] In attempting to directly measure the tpa antigen level in whole plasma using the ELISA technique of Bergsdorf et al,[15] however, we found that the apparent level of tpa antigen was not independent of the dilution of plasma assayed, with higher dilutions producing higher apparent tpa antigen levels (data not shown). In contrast, use of euglobulin precipitates resulted in tpa antigen levels that were independent of the dilution of euglobulin fraction assayed. In agreement with other investigators[16] we also found that purified tpa added to plasma was quantitatively recovered in the euglobulin precipitate over a wide range of concentrations. Thus, it is likely that both free tpa and tpa bound to the inhibitor are detected with equal efficacy in the euglobulin fraction using the ELISA of Bergsdorf et al.[15]

There is as yet no agreement on the optimal method for measuring tpa inhibitor activity in plasma. Current assays are not standardized and may have methodologic limitations.[22] The method we have employed, in which a single concentration of purified tpa is added to plasma, has been criticized on the grounds that the amount of tpa inhibitor activity in plasma increases as increasing amounts of tpa are added.[22] Nonetheless, the finding that tpa inhibitor by this assay correlates strongly with both overall fibrinolytic activity and free tpa activity in patients with cirrhosis supports its usefulness in evaluating tpa inhibitory capacity of plasma. This type of tpa inhibitor assay has also proven useful in the

Table 2. Fibrinolytic Assays (Mean ± SD)

	Group 1	Group 2	Group 3	Normal
DWBCLT (minutes)	104 ± 35	176 ± 26	>240	>240
TPA antigen (ng/mL)	29.4 ± 25.9	30.9 ± 20.7	22.1 ± 13.9	5.2 ± 2.6
TPA activity (ng/mL)	7.4 ± 12.1	0.1 ± 0.2	0	0
TPA inhibitor (ng/mL)	16.2 ± 10.5	25.3 ± 15.8	32.0 ± 18.7	41.9 ± 9.8
a2PI (%)	53 ± 22	52 ± 25	77 ± 26	73-119%

study of impaired fibrinolysis in atherosclerotic heart disease.[23]

Tpa inhibitors appear to be synthesized by endothelium, platelets, and placenta.[24-26] Normal plasma may contain both endothelial and nonendothelial tpa inhibitors[27]; the assay method of Juhan-Vague et al[17] measures only the total tpa inhibitory activity in plasma and does not distinguish among the various inhibitors present. Thus, we cannot determine which type of tpa inhibitor is most important in the regulation of fibrinolytic activity in patients with liver cirrhosis. Moreover, since the factors controlling tpa inhibitor synthesis and release are not well defined, it is not yet possible to explain why some patients with cirrhosis are able to counteract increased levels of circulating tpa with increased levels of inhibitor, while others are not. Studies of the extent of protein C activation in cirrhosis may be relevant, since there is evidence that activated protein C inhibits tpa inhibitor release from cultured endothelium.[28] Thrombin may also be an important factor, since subclinical disseminated intravascular coagulation is common in liver cirrhosis,[29,30] and thrombin has been shown to stimulate release of both tpa antigen and inhibitor by cultured endothelium.[31]

We found mean a2PI to be significantly reduced in patients with cirrhosis and accelerated fibrinolysis and normal in patients with cirrhosis and normal fibrinolysis, suggesting a significant contributing role for reduced a2PI in the pathogenesis of accelerated fibrinolysis in cirrhosis. Although a2PI did not correlate with the DWBCLT, the DWBCLT method we used may be insensitive to the inhibitory activity of a2PI, since it involves clotting whole blood in the absence of calcium, thus interfering with the cross-linking of a2PI to fibrin by factor XIII.[32] In contrast to a previous study,[8] we also found no correlation between a2PI and serum albumin, suggesting that factors other than hepatic synthetic rate are important in determining the level of a2PI in cirrhosis.

In addition to increased tpa activity, the possible contribution of increased urokinase-like plasminogen activator activity[7] to the pathogenesis of accelerated fibrinolysis in cirrhosis needs to be considered. Since tpa inhibitors have also been reported to inhibit urokinase,[9,17,24,26] reduced tpa inhibitory activity in cirrhosis may explain the apparently conflicting reports of increased urokinase-like activator activity[7] and normal urokinase antigen[33] in patients with cirrhosis.

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Ki-M8 Monoclonal Antibody Reactive With an Intracytoplasmic Antigen of Monocyte/Macrophage Lineage

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A monoclonal antibody (MoAb), Ki-M8, that reacts specifically with cells of the monocyte/macrophage system is described. On light and electron microscopic immunohistochemistry, Ki-M8 recognizes intracytoplasmatically localized antigens of mol wt 30,000 and 32,000, increasingly expressed during differentiation of monocytes into macrophages. Ki-M8 antigen is detectable on almost all known tissue macrophages and monocyte/macrophage-related cell lines after appropriate stimulation. In functional terms Ki-M8 significantly impairs the generation of oxygen radicals during an induced respiratory burst. Applied to acute nonlymphoblastic leukemias, a clear-cut differentiation of the monocytic phenotype and differentiation is possible on the basis of Ki-M8 immunoreactivity. Ki-M8 represents a reagent specific for the monocyte/macrophage system with regard to antigen distribution in normal and neoplastic cells as well as with regard to its influence on a typical monocyte/macrophage-related function.

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AMONG THE LARGE number of monoclonal antibodies (MoAbs) in current use, only a few have achieved functional relevance, especially those reacting with cells involved in nonspecific defense and immune response. This issue is best exemplified by MoAbs to immunoglobulins of B lymphocytes and by those recognizing histocompatibility antigens[1-6] or surface antigens of T cell subpopulations.[7,8] In addition to antigen presenting[9] and phagocytosis,[10] cells belonging to the monocyte/macrophage system have attracted considerable interest due to their secretory activities and generation of oxygen radicals.[11,12] The latter function is associated with the respiratory burst and is correlated with ingestion and killing of microorganisms, which require a close interaction between cytoplasmic organelles, notably lysosomes, and membrane structures.

In the present study we describe a MoAb, termed Ki-M8, that, in contrast to hitherto known MoAbs directed to surface membrane structures,[13-15] recognizes the bulk of lysosomal membranes of cells of the monocyte/macrophage system under normal and neoplastic conditions. In functional terms Ki-M8 significantly diminishes the generation of oxygen radicals during the respiratory burst as measured by chemiluminescence, whereas particle ingestion is not affected.

MATERIALS AND METHODS

Generation of Monoclonal Antibody

Macrophages were obtained by glass adherence from fresh minced human lymph node specimens, which were removed because of enlargement due to chronic lymphadenitis and used as immunogen. Splenic cells of Balb/c mice were fused with the nonsecreting myeloma cell line X63-Ag8.653 at a ratio of 1:1.[16] After suspension in HAT-supplemented culture medium (1×10^{-4} mol/L hypoxanthine, 4×10^{-7} mol/L aminopterin, 6×10^{-5} mol/L thymidine; Boehringer, Mannheim, West Germany), cells were dispersed in Greiner 24-well fusion plates (Greiner, Nürtingen, West Germany) at a concentration of 1×10^6 cells/well using 1×10^5 syngeneic peritoneal macrophages per well as feeder layer. After about ten days, when viable cell clusters were visible, supernatants were screened for immunohistochemical reactivity using cryostat sections of human tonsils (see below). The hybridoma colony showing the Ki-M8 specificity as described in this study was subjected to a limiting dilution procedure and processed for mass culturing and ascites production. On immunodiffusion, MoAb Ki-M8 was shown to be of IgG1 type.

Specificity Tests of the MoAb Ki-M8

Light microscopic immunohistochemistry. Immunohistochemical staining was performed on 8 μm cryostat sections of surgically removed tissue specimens. Special attention was given to those organs known to be populated by cells of the monocyte/macrophage system (n = 4 for each analyzed organ). Furthermore, cytospin preparations of normal human granulocytes (n = 5), T lymphocytes (n = 5) and B lymphocytes (n = 5), monocytes (n = 5), peritoneal (n = 3) and alveolar macrophages (n = 4) separated as described elsewhere,[17,18] and smears of bone marrow (n = 3) were subjected to immunohistochemical testing. Because of the close association of Ki-M8 reactive cells to T cell areas of lymphoid tissue (see results), special attention was given to the purification of T cell populations by passage of nonadherent cells of the interphase through an antihuman immunoglobulin column packed with beads coupled with human IgG, antihuman rabbit IgG, and human C3, as described in detail elsewhere.[17] In addition, Ki-M8 reactivity was studied on monocytes cultured over five days by prolonged glass adherence (n = 3) as well as stimulated over five days with lymphokine-conditioned media (n = 3) obtained by addition of concanavalin A (Con A; Serva, Heidelberg, West Germany; 30 μg/mL) to separated normal blood lymphocytes over three days.[18] The following cell lines were tested: Unstimulated and 12-0-tetradecanoylphorbol-13-acetate stimulated U-937 cells (TPA; Sigma, Munich, West Germany; 1.6×10^{-8} mol/L over three days); unstimulated; dimethyl sulfoxide (DMSO)-stimulated (1.2% over five days); and TPA-stimulated HL-60 cells (1.6×10^{-8} mol/L over three days). These cell lines represent promyelocytes (HL-60), monocyte precursors (U-937), and, following stimulation, mature granulocytic (DMSO stimulation of HL-60) or monocyte/macrophage differentiation forms (TPA stimulation of HL-60 and U-937).[19-21] In addition, cell lines BALM4, NALM6, BALL-1 (considered as B cell leukemia lines), cell lines CCRF-CEM, SKW-3, TALL-1 (considered as T cell leukemia lines), cell lines KG-1, ML1, (considered as myelomono-

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cytic leukemia lines), and the non-T, non-B leukemia cell lines K-562 and Reh[22] were tested for immunoreactivity with MoAb Ki-M8.

The immunoperoxidase method was applied as described in detail elsewhere[23,24] using a three-step incubation procedue with MoAb Ki-M8 (undiluted culture supernatants or 1:2,000 diluted ascites fluid). To avoid endogenous peroxidase activity and to increase sensitivity, the immune alkaline phosphatase technique was applied in some cases.[25] To exclude surface binding of MoAb antibody Ki-M8, cell suspensions of unstimulated monocytes and monocytes stimulated by lymphokine-conditioned media and zymosan A (see below) were stained by indirect immunofluorescence. After incubation with MoAb Ki-M8 (60 minutes, 4°C) and intensive washing, rhodamine-conjugated goat antimouse IgG (Medac, Hamburg, West Germany) was added, (60 minutes, 4°C) and, following two washing steps, cells were evaluated by fluorescence microscopy.

Electron microscopic immunohistochemistry. Electron microscopic immunohistochemistry was performed on 1- to 3-mm slices of lymph node specimens fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) for one hour and snap frozen in liquid nitrogen. Cryostat sections were then used for the immunoperoxidase method as described above followed by postfixation in 1% osmium tetroxide in 0.1 mol/L phosphate buffer, pH 7.2.[26,27]

As positive controls we used the commercially available MoAb antimonocyte 1 (Mono 1[14]; Bethesda Research Laboratory, Gaithersburg, MD). As negative controls, supernatants of unfused myeloma cells and sera of nonimmunized mice (instead of the primary MoAb Ki-M8) were applied, which did not render any immunoreactivity pattern and thus excluded nonspecific binding during the immunohistochemical procedures.

Functional and Biochemical Analyses

Measurement of chemiluminescence was performed as described by Kato et al.[28] Twenty micrograms luminol (Sigma; dissolved in 10 μL PBS) was added to 500 μL monocyte suspension (2×10^6 cells/mL RPMI 1640). After preincubation for ten minutes at 37°C, 1 mg zymosan A (Sigma) dissolved in 20 μL medium was added and the chemiluminescence response was recorded over 40 minutes using the Berthold Biolumate Counter model LB 9505 (Wildbad, West Germany). Chemiluminescence was measured in untreated monocytes and monocytes to which monoclonal antibody Ki-M8 was added in a dilution of 1:100, 1:500, 1:1,000, and 1:2,000 for 2.5 hours. In addition, chemiluminescence measurement was performed for untreated monocytes and monocytes treated with MoAbs Ki-M8 and antimonocyte 1 (diluted 1:100) for 2.5 hours and 24 hours. Furthermore, chemiluminescence response of granulocytes was checked over 40 minutes after incubation with MoAb Ki-M8 (diluted 1:100) in comparison to untreated granulocytes.

For the estimation of the mol wt of the Ki-M8–recognized antigen, proteins were extracted from freshly ectomized human tonsils (n = 3) with Triton-X-100 (1 vol% in aqua dest) and solubilized in sample buffer (0.125 mol/L Tris buffer, pH 6.8, containing 4% sodium dodecyl sulfate (SDS) and 20 vol% glycerin). After incubation for two hours at room temperature, electrophoresis was performed under nonreducing conditions on a 0.3-cm thick 8% SDS-polyacrylamide gel over 7-cm separation distance according to Laemmli.[29] Proteins were blotted overnight at 4°C on nitrocellulose membranes (Schleicher and Schüll, Dassel, West Germany).[30] Membranes were immunostained applying the immune alkaline phosphatase method as described above using a mouse MoAb against alkaline phosphatase of calf intestine as tertiary antiserum after covering of unspecific binding sites. Immunostained protein bands were compared with the whole tonsillar protein pattern and a mol wt standard stained with Amido black.

Analysis of Leukemias

Fresh heparinized venous blood of 40 patients with acute leukemias was subjected to Ficoll-Urografin density gradient centrifugation at d = 1.077 g/mL.[31] Cytocentrifuge preparations of the interphase consisting of leukemic blasts, myeloid precursors, as well as normal lymphocytes and monocytes, were subjected to Pappenheim staining and enzymecytochemical staining for peroxidase,[32] naphthol AS-D chloroacetate esterase,[33] and nonspecific acid esterase.[34] Immunophenotyping was performed with the immune alkaline phosphatase method as described above. In additon to Ki-M8, the following MoAbs were applied: Vim 2 specific for granulocytes and myelomonocytic precursors and Vim D5 specific for granulocytes[35,36]; My 7-recognizing granulocytes, monocytes, and myelomonocytic precursors[37]; 3C4 described by Schienle et al[38] specific for granulocytes and granulocytic precursors; Ki-M1 specific for monocytes.[39] The lymphoblastic nature of the analyzed leukemias was excluded by their negative reaction for the MoAbs To15 and HD37 used as pan-B cell reagents[40,41] as well as by their negative reaction for the MoAb Leu-4 used as pan-T cell reagent.[42] In addition, Ki-M8 was applied to 30 cases of acute and chronic lymphocytic leukemias diagnosed on the basis of morphological and enzymecytochemical criteria and on the basis of their reactivity with the lymphoid-associated MoAbs mentioned above.

RESULTS

Reactivity Pattern

Light microscopy. On light microscopic immunohistochemical testing, Ki-M8 reacted with an intracytoplasmatically localized antigen selectively expressed on cells of the monocyte/macrophage system. The failure of Ki-M8 to recognize surface structures could also be confirmed by indirect immunofluorescence staining of viable unstimulated monocytes and monocytes stimulated with lymphokine-conditioned media, which did not show any surface labeling. Considering separated cell populations, blood monocytes displayed the lowest degree of reactivity, becoming faintly visible only after applying the immune alkaline phosphatase technique. On the contrary, in stimulated blood monocytes as well as peritoneal and alveolar macrophages, reactive cytoplasmic structures could only hardly be discriminated because of the high degree of reactivity (Fig 1). Granulocytes as well as T and B lymphocytes did not reveal any positive immunohistochemical staining by counting 500 cells in each sample. Special attention was given to granulocytes that did not render any positive staining of either intracytoplasmic or surface structures. The exclusive reactivity with the monocyte/macrophage system could also be shown by testing permanent cell lines. Only those that were induced to differentiate along the monocyte/macrophage differentiation pathway, such as TPA stimulated HL-60 and U-937 cells, proved to be positive for Ki-M8. Unstimulated myelomonocytic leukemia lines as well as B cell, T cell, and non-T, non-B cell leukemia lines were clearly negative for Ki-M8 (Table 1).

Studying different tissues all known macrophage populations, with the exceptions of starry sky macrophages of lymphoid follicles (Fig 2) and macrophages of central nervous tissue, reacted with Ki-M8. The reactive cell populations included macrophages of lymph node sinuses and

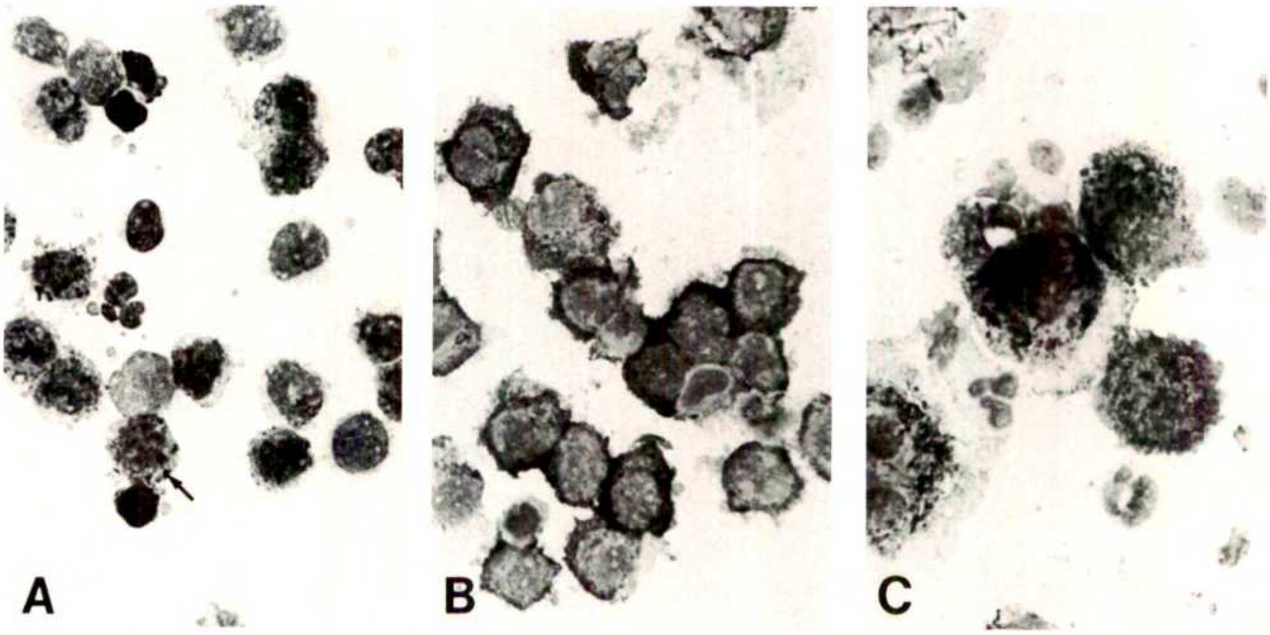

Fig 1. Ki-M8 reactivity in separated normal human monocytes (A), lymphokine-stimulated monocytes (B; for details see text), and alveolar macrophages (C). There is a stepwise increase of immunoreactivity from unstimulated blood monocytes (faint granular pattern; arrow) to alveolar macrophages (strong diffuse reactivity). Cytospin preparations, immune alkaline phosphatase reactions, original magnification × 560.

Table 1. Reactivity Pattern of Ki-M8 as Tested by Immunohistochemistry on Separated Blood Cells, Cell Lines, and Tissue Samples

Granulocytes	–
B and T lymphocytes	–
Monocytes, unstimulated	(+)
Monocytes, stimulated*	+
Myelomonocytic leukemia lines*	–
HL-60 and U-937, stimulated*	+
B cell, T cell, and non-T, non-B leukemia lines*	–
M of lymphoid follicles (starry sky M)	–
M of lymphatic T zones	+
M of lymph node sinuses	+
M of bone marrow	+
M of splenic red pulp	+
M of liver (von Kupffer cells)	+
Peritoneal and alveolar M	+
M of connective tissue (histiocytes)	+
M of gastrointestinal tract	+
M of placenta (Hofbauer cells)	+
M of renal interstitium	+
M of brain (glial cells)	–
Dendritic reticulum cells	–
Interdigitating reticulum cells	–
Langerhans' cells	–

Abbreviations: –, negative; (+), weakly positive; +, positive; M macrophages.

*See text for details.

interfollicular macrophages of lymphoid tissue (lymphatic T-zones), monocytoid-differentiated mononuclear cells and macrophages of bone marrow, macrophages of splenic red pulp, von Kupffer cells of the liver, macrophages of connective tissue known as histiocytes, macrophages of the gastrointestinal tract and interstitium of the kidney, as well as Hofbauer cells as the macrophage population of the placenta (Table 1). Dendritic reticulum cells (follicular dendritic cells) of lymphoid follicles (Fig 2) as well as interdigitating reticulum cells of lymphatic T-zones and Langerhans' cells of the epidermis, known as accessory cells of B cell and T cell immune response, respectively, and differently claimed to belong to the monocyte/macrophage system, were clearly negative for Ki-M8 (Table 1). The reactivity of Ki-M8 with natural killer (NK) cells seemed to be unlikely because immunohistochemical staining of lymphoid tissue with Leu-7[43] (Becton Dickinson, Mountain View, CA) specific for NK cells clearly revealed a different staining pattern of tonsils with the bulk of reactive cells within the follicle. Cross-reactions with other tissue structures could not be observed, such as those of epithelial, endothelial, neural, and mesenchymal origin analyzed in organs mentioned in Table 1, in endocrine organs, in male and female genitals, in salivary glands, and in lung, heart, and striated muscle.

Electron microscopy. On electron microscopic evaluation, the reactivity of Ki-M8 was mainly confined to the bulk of lysosomal structures as well as phagosomes. Within these organelles Ki-M8 stained the surrounding walls as well as part of the interior. Other cell structures, especially the surface membrane and nuclei, did not show any immunoreactivity (Fig 3).

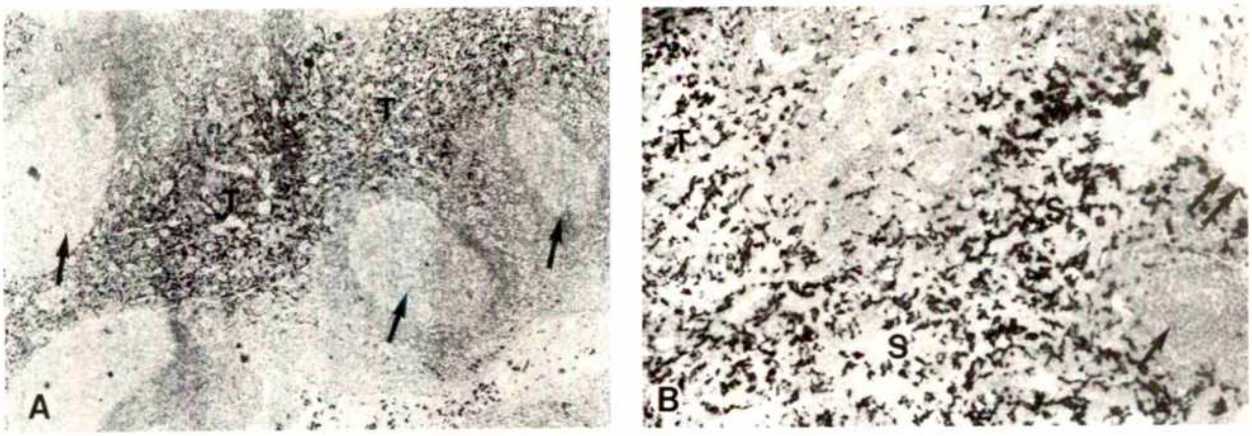

Fig 2. Light microscopic immunohistochemical reactivity of Ki-M8 in human tonsil (A) and lymph node (B). Lymphoid follicles populated by starry sky macrophages and dendritic reticulum cells are negative for Ki-M8 (arrow). Interfollicular macrophages of lymphatic T zones (T) and macrophages of lymph node sinuses (S; double arrow = lymph node capsule) show strong reactivity. Cryostat sections, immunperoxidase reactions, original magnification × 140 (A), original magnification × 350 (B).

Results of Functional and Biochemical Analyses

Blood monocytes treated for 2.5 hours with Ki-M8 showed a significantly decreased chemiluminescence response (Fig 4A). Whereas dilutions of Ki-M8 of 1:2,000 did not show any significant effects, dilutions of down to 1:100 lead to a dose-dependent decrease of chemiluminescence response amounting to about 30% in case of 1:100 dilution as compared with untreated and antimonocyte 1-treated monocyte samples (Fig 4B and C). These differences could not be due to variations or undesired stimulation during monocyte separation because one and the same blood sample was used for these experiments. After prolonged culture over 24 hours, no further differences could be observed between the untreated and Ki-M8 as well as antimonocyte 1-treated monocytes (Fig 4C). Particle ingestion was not influenced by either Ki-M8 or antimonocyte 1 (data not shown). In addition, as shown by light microscopic immunohistochemistry and immunofluorescence staining, phagocytosis of zymosan A did not induce surface expression of the Ki-M8 antigen.

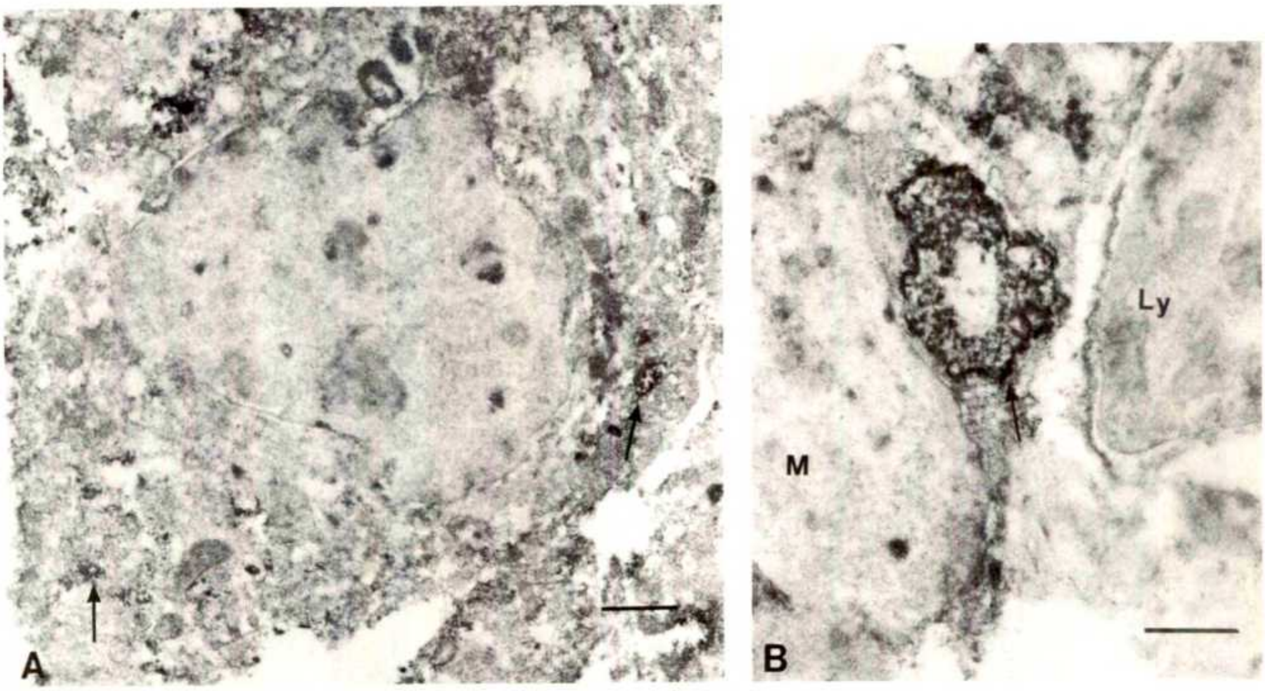

Fig 3. Electron microscopic immunohistochemical reactivity of Ki-M8 in macrophages of human lymph node. Ki-M8 reaction site is confined mainly to lysosomes and vesicular structures (A; arrow) as well as phagosomes (B; arrow; Ly = lymphocyte, M = macrophage). Paraformaldehyde fixation, immunoperoxidase reactions, postfixation with osmium tetroxide, resin embedding, original magnification × 13,500 (A), original magnification × 16,500 (B); bar = 1 μm.

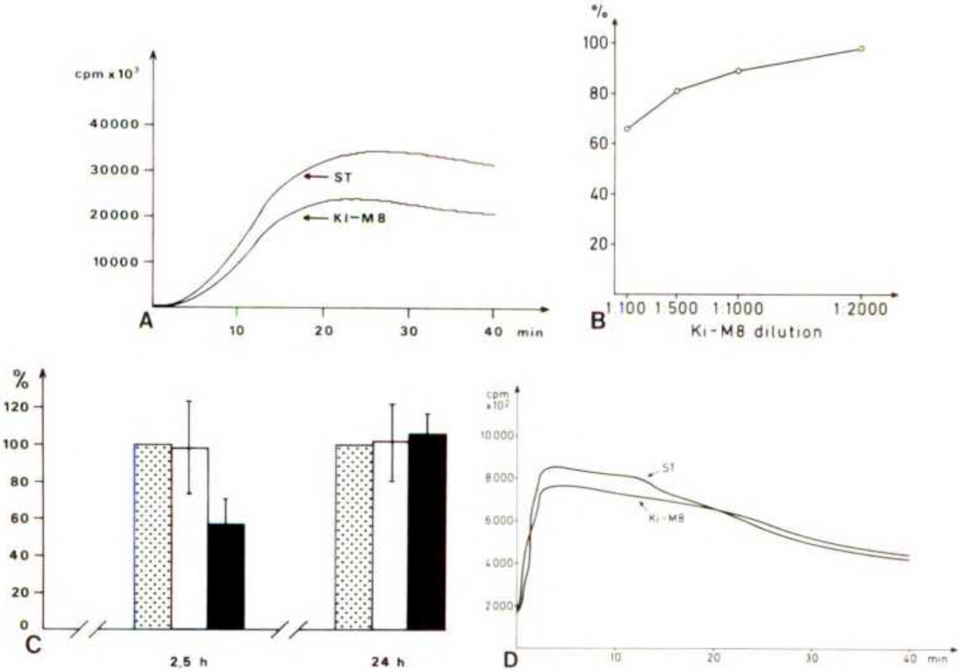

Fig 4. (A) Chemiluminescence response (cpm × 10³) of blood monocytes treated with MoAb Ki-M8 over 2.5 hours. Ki-M8 significantly inhibits the generation of oxygen radicals measured over 40 minutes in comparison to untreated blood monocytes (standard = ST). (B) Chemiluminescence response of blood monocytes treated with various dilutions of MoAb Ki-M8 over 2.5 hours (mean values, n = 4). Chemiluminescence of untreated blood monocytes corresponds to 100%. A dilution of 1:100 of MoAb Ki-M8 leads to a decrease of chemiluminescence amounting to about 30%. (C) Chemiluminescence response (cpm × 10³) of blood monocytes treated with MoAbs Ki-M8 and antimonocyte 1 (Mono 1) over 2.5 and 24 hours (n = 4) in comparison to untreated blood monocytes (standard = ST = 100%). A significant inhibition of chemiluminescence response can be observed only in case of monocytes treated with Ki-M8 over 2.5 hours. ■ KI – M8; ☐, MONO 1; ▨, ST. (D) Chemiluminiscence response (cpm × 10²) of untreated (standard = ST) and Ki-M8-treated granulocytes measured over 40 minutes. A significant difference cannot be detected.

Chemiluminescence responses of granulocytes, untreated or treated with Ki-M8, did not show significant differences (Fig 4D).

Ki-M8 immunostained two protein bands of 30.000 and 32.000 daltons under nonreducing conditions (Fig 5). These protein bands could be observed in all three samples of tonsilar extracts analyzed, although with different degrees of intensity. Control stainings applying physiologic saline or supernatants of unfused myeloma cells instead of MoAb Ki-M8 did not show any positively stained protein band (data not shown).

Ki-M8 in Myelomonocytic Leukemias

The number of blasts in the analyzed cases ranged from 29% to 97%. Blasts were considered as reactive with a given MoAb antibody if the number of positive blasts exceeded 10%. Ki-M8 showed a positive immunostaining in eight of the analyzed cases (20%), which were also reactive with the MoAbs My7- and Ki-M1-recognizing monocytes but not with the MoAbs Vim2-, VimD5-, and 3C4-recognizing granulocytes and myelomonocytic precursors. Based on these data a clear distinction between the monocytic, on one hand, and the pure granulocytic and myelomonocytic leukemias, on the other hand, could be obtained. Enzymecytochemically Ki-M8 reactive cases were positive for peroxidase, naphthol AS-D chloroacetate esterase, and nonspecific acid esterase in eight (100%), four (50%), and eight (100%) cases, respectively. Thus a direct relationship between the immunohistochemical analysis and the French-American-British (FAB) classification applying morphological and enzymecytochemical criteria[44] could not be found, as shown also by other authors.[45,46] The incidence of cases with a positive reaction to

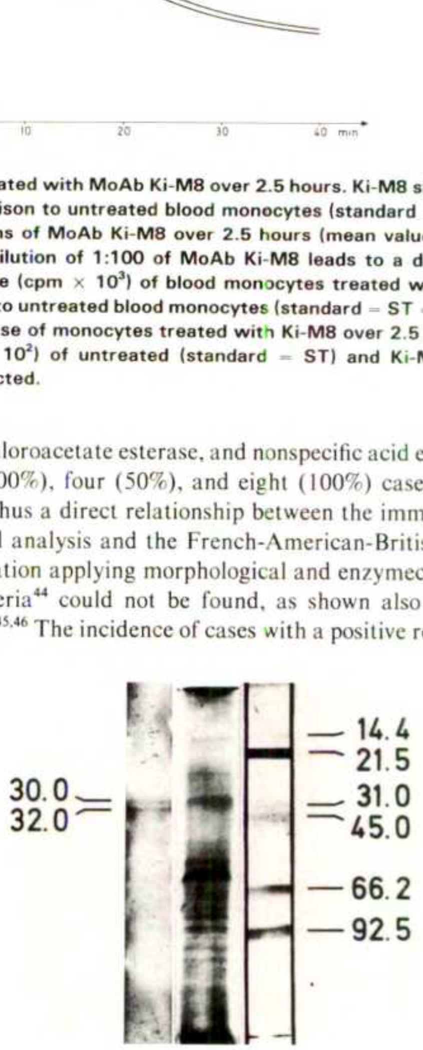

Fig 5. Antigens immunostained by the MoAb Ki-M8 in comparison to the whole tonsillar protein pattern and a mol wt standard stained with Amido black (from left to right; for details see text). The antigens display mol wt of 30,000 and 32,000 daltons.

these enzymecytochemical stainings among the remaining 32 patients amounted to 19 (60%), eight (25%), and 31 (97%), respectively. The analyzed cases of acute and chronic lymphocytic leukemias did not show any immunoreactivity with MoAb Ki-M8.

DISCUSSION

The significance of monocytes and macrophages for the immune response[47,48] and the multitude of disorders caused by abnormalities of these cells[49,50] underline the importance of reliable reagents characterizing this cell system. Monocytes and macrophages have been defined by morphological, cytochemical, immunologic, and functional methods[51,52]; most of such analyses, however, have been performed in vitro using easily accessible members of this cell system such as blood monocytes and macrophages of serous cavities in addition to permanent monocyte/macrophage-related cell lines subjected to different stimulation procedures. This issue also holds true for most of the MoAbs established so far to characterize the monocyte/macrophage system.[13-15,53,54] The majority of such reagents did not prove entirely specific due to their concomitant reactivity with granulocytes,[13,55] T lymphocyte subpopulations,[56-59] including NK cells,[60] or platelets.[55]

The MoAb Ki-M8 described in this study fulfills the criteria of a restricted occurrence within the monocyte/macrophage system applying samples of separated cell populations, of human tissue sections, and of permanent human cell lines. On immunohistochemical staining of frozen tissue sections and smears of bone marrow, only monocytes and macrophages with the typical distribution pattern in lymphatic and nonlymphatic organs were selectively recognized (Figs 1 and 2). The only exception was made by the so-called starry sky macrophages of the lymphoid follicles and macrophages of the central nervous tissue. Antigen-presenting cells (accessory cells of B cell and T cell immune response) such as dendritic reticulum cells (follicular dendritic cells)[61] of lymphatic B zones as well as Langerhans' cells of the skin and interdigitating reticulum cells of lymphatic T zones[62] were not immunostained by Ki-M8, although their relationship to the monocyte/macrophage system has variously been claimed.[24,39,63] Considering permanent human cell lines, the promyelocytic cell line HL-60 and the histiocytic cell line U-937 revealed positive only after adequate stimulation to evolve into mature macrophages (Table 1). Epithelial, endothelial, and neural cells as well as mesenchymal cells not related to macrophages, including lymphocytes and granulocytes, invariably showed a negative reaction.

Considering the fundamental capabilities of monocytes/macrophages and related cells beyond their ability to differentiate from bone marrow precursors over monocytes into tissue macrophages[64] and beyond their regulatory function during immune response[47,48] mainly expressed in antigen presentation, the effector function in connection with nonspecific defense against microorganisms has been extensively investigated. Tests covering these aspects of macrophage function measure phagocytosis and release of lysosomal contents.[10-12] The ultrastructural localization of Ki-M8 reactive antigen in lysosomal structures (Fig 3) motivated us to study the effect of Ki-M8 on such effector functions usually considered to be bound to lysosome integrity. The results showed that Ki-M8 significantly impaired the generation of oxygen radicals during the respiratory burst primed by ingestion of zymosan particles, whereas particle incorporation was not influenced (Fig 4A and B). Other monocyte/macrophage specific MoAbs of the same immunoglobulin class recognizing exclusively surface antigens such as anti-monocyte 1[14] did not show any effect on this function (Fig 4C). The observation that Ki-M8 impaired respiratory burst leaving particle incorporation unaltered may suggest a direct effect of Ki-M8 on oxygen-radical generating mechanisms rather than on phagocytosis activity or on modulation of membrane receptors. It remains to be established, however, by which mechanism Ki-M8 is internalized in monocytes during incubation to exert effects on chemiluminescence and why this effect is abolished after 24 hours.

In a further step we applied the MoAb Ki-M8 to acute nonlymphoblastic leukemias. In the FAB classification of monocytic and granulocytic leukemias in addition to the pure granulocytic (M1-M3) and pure monocytic differentiation patterns (M5), a further group of leukemias having granulocytic as well as monocytic properties in common (M4) has been separated.[44] These myelomonocytic leukemias (M4) probably reflect frozen differentiation stages of precursor cells with a bimodal differentiation potency to evolve either in granulocytes or monocytes. Considering the fact that myelomonocytic precursors may express nonspecific acid esterase, there is a need for a more specific marker to clearly separate the monocyte lineage. The high specificity of Ki-M8 for monocytic cells and specifically its negativity in all three types of granulocytes render this antibody appropriate for the application under this aspect. In 40 cases of acute nonlymphoblastic leukemias, eight cases were found to possess the monocytic phenotype due to their characteristic pattern of reactivity being positive for Ki-M8 and being negative for MoAbs recognizing granulocytes and myelomonocytic precursors.

There seems to be no evidence that the Ki-M8 reactivity pattern could be covered by one of the hitherto described MoAbs directed against the monocyte/macrophage system, since antigens with mol wt of 30,000 and 32,000 (Fig 5) and with an intracytoplasmatic localization (Fig 3) have not been described so far for this cell system.[13-15,39,53-60,65-69] Some similarity might exist to the MoAb recently established by Zwadlo et al[69]; in contrast to this reagent the Ki-M8 antigen is weakly expressed in blood monocytes and increases mainly during the late stages of monocyte/macrophage differentiation. To our knowledge Ki-M8 comprises one of the first MoAbs specific for monocytes and macrophages not only on the basis of antigen distribution under normal and neoplastic conditions but also considering its effect on a typical monocyte/macrophage-related function. Further studies will show whether the inhibitor potency of Ki-M8 will also apply for other functions being probably dependent on lysosomal integrity such as other secretory activities[70] or lysis of tumor cells.[71,72]

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Lactoferrin Derived From Neutrophils Inhibits the Mixed Lymphocyte Reaction

By Kevin Slater and John Fletcher

The effects of the products of phagocytosing neutrophils on lymphocyte transcriptional activity have been investigated using allogeneic mixed lymphocyte cultures (MLC). When MLCs are grown crowded in round-bottomed wells, uptake of tritiated uridine is inhibited by the presence of neutrophil products. Similarly, inhibitory activity is shown by purified lactoferrin. The inhibitory factor released by neutrophils is positively identified as lactoferrin, since its activity is lost in the presence of specific antibody. In addition, inhibitory activity is lost when lactoferrin is removed by a monoclonal antibody (MoAb) column and is recovered with lactoferrin from the column. The active form of lactoferrin carries iron and the apoprotein is inactive. MLCs crowded in round-bottomed wells take up considerably more uridine than when spread in flat-bottomed wells, and it is this enhanced transcriptional activity that is inhibited by neutrophil-derived lactoferrin. Enhanced uridine uptake by crowded cultures is due to factors that can be transferred in the supernatants to promote uridine uptake by spread cultures. Neutrophil lactoferrin inhibits both production and effect of the transferable factor(s).

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A NUMBER OF ROLES have been suggested for the iron binding protein lactoferrin (Lf), usually in relation to host defenses, since the binding of iron to apo-Lf makes it unavailable to invading organisms and limits their growth.[1] This is probably its function in the many secretions in which it is found in high concentrations, including milk, tears, gastric and bronchial secretions, and seminal fluid.[2] Its role in plasma is less clear, as the plasma protein transferrin is quantitatively much more important in binding iron and limiting its availability to microorganisms.[3] However, Lf binds iron at lower pH than transferrin and may take over a bacteriostatic function in the acidic conditions of pus.[4]

Plasma lactoferrin is derived from the secondary granules of neutrophils and is discharged from the cell when neutrophils are activated during inflammation.[5,6] A number of extracellular functions influencing the inflammatory response have been described, including aggregation of neutrophils,[7] inhibition of the complement system,[8] suppression of antibody production,[9] modulation of natural killer (NK) cell activity,[10] and inhibition of colony-stimulating-factor (CSF) production.[11] The inhibition of CSF production may be a direct action of Lf on monocytes and macrophages or an indirect action by inhibition of a monokine required for CSF production from T lymphocytes, fibroblasts, and vascular endothelial cells.[12-14] Thus both the effects upon CSF production and on antibody production indicate an interaction between Lf and mononuclear cells involving control of protein synthesis. There is some evidence for a similar action of neutrophil products in vivo, as patients with peripheral blood neutrophil counts greater than $15 \times 10^9\ L^{-1}$ showed impaired skin responses to various T and B mitogens; normal responses return when neutrophil counts fall.[15] The hypothesis that lactoferrin released from neutrophils may have a role in control of lymphocyte responses has been investigated using an allogeneic mixed lymphocyte culture (MLC) that allows measurement of factors in the culture supernatants in the absence of stimulatory substances such as lectins and exogenous endotoxins. We have shown that a factor released from phagocytosing neutrophils inhibits transcriptional activity in lymphocyte cultures, identified the factor as lactoferrin, and demonstrated a requirement for iron to activate the molecule.

MATERIALS AND METHODS

Subjects. Blood was obtained from normal, healthy volunteers among the laboratory staff.

Neutrophils. Peripheral blood was taken into EDTA and the neutrophils separated by centrifugation over a Mono-Poly Resolving Medium (Flow Laboratories, Rickmansworth, UK). The cells were washed three times in phosphate-buffered saline (PBS) free of Ca^{2+} and Mg^{2+}. The cells were then resuspended in PBS containing 1 mmol/L $CaCl_2$, and 0.7 mmol/L $MgCl_2$ at a final cell concentration of $2 \times 10^6\ mL^{-1}$.

Phagocytosis. *Candida guilliermondii* were cultured overnight in glucose broth and then heat killed at 100°C for 15 minutes. The cells were washed and opsonized in pooled human serum for 45 minutes at 37°C with continuous mixing. The *C guilliermondii* were resuspended at $10^7\ mL^{-1}$ in PBS containing Ca^{2+} and Mg^{2+}.

Equal volumes of the neutrophil and *C guilliermondii* preparations were mixed together and incubated with continuous mixing at 37°C for 30 minutes. The cells were removed by centrifugation and the supernatant sterilized by passage through a micropore filter (Flow Laboratories). Aliquots of the "phagocytosing neutrophil-conditioned medium" (PNCM) were stored at −20°C until required.

Lactoferrin. Lactoferrin (Lf) from human breast milk was obtained from Sigma Chemical Co, St. Louis. This was prepared in Tris/HCl buffer and used either iron free (Apo-Lf) or 50% iron saturated (Fe-Lf). Fifty percent iron saturation was achieved according to the method of Gutteridge et al[16] to avoid nonspecific binding. Briefly, Lf was prepared in 0.2 mol/L Tris/HCl buffer containing 10 mmol/L $NaHCO_3$, and iron was added as $Fe_2(NH_4)_2(SO_4)_2$ taking 1.4 µg Fe per mg of protein to give 100% saturation. The iron and protein were incubated at 37°C for 30 minutes. Fifty percent saturation was confirmed by detection of an absorbance peak at 460 nm. Sigma Lf is stated to be 0.1% iron saturated, but no absorbance could be detected at 460 nm. The Lf preparations were filter sterilized and aliquots stored at −20°C until

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required. The Lf concentration of PNCM was kindly measured by Dr J. Porter of University College Hospital, London using an enzyme-linked immunosorbent assay (ELISA) technique.

Antibodies. Rabbit antihuman breast milk Lf was obtained from Dako-Patts, High Wycombe, UK. Monoclonal anti-Lf against human neutrophil Lf was a generous gift Dr J. Brock (Glasgow University).

The antibodies were bound to CNBr-activated Sepharose 4B according to the method of van Eijk.[17] The efficacy of the antibody columns was determined by passage of ^{59}Fe-labeled Lf down the columns. This could be completely retained and subsequently recovered using Glycine/HCl buffer at pH 2.8. Detection of an absorbance peak at 460 nm indicated that this treatment did not cause dissociation of the bound iron.

Mixed lymphocyte cultures. Peripheral blood from three volunteers was taken into heparin and the mononuclear cells obtained by centrifugation over lymphocyte separation medium (LSM) (Flow Laboratories). The cells were washed three times in RPMI 1640 and resuspended at 1.1×10^6 mL^{-1} in RPMI containing 15% fetal calf serum, nonessential amino acids, penicillin, streptomycin, and 10^{-6} mol/L indomethacin (c-RPMI). The cells from the three donors were kept separate throughout and were only mixed prior to use. One hundred eighty microliters of the mixed lymphocyte culture (MLC) was added to the wells of 96-well tissue culture plates (Costar, Cramlington, UK). Twenty microliters of Lf or PNCM dilution was added in quadruplicate; thus standard conditions consisted of 2×10^5 cells in 200 μL. Spread cultures and crowded cultures were produced in flat-bottomed and round-bottomed wells respectively. The cultures were incubated at 37°C at 100% relative humidity with 5% CO_2 in air for 72 hours unless otherwise stated.

To determine the direct effect of Lf and PNCM on the MLC, 0.2 μCi tritiated uridine (^{3}H-UdR) in 20 μL was added to the cultures for the last six hours of the culture period. The cells were then harvested onto glass fiber mats using a Dynatech Automash 2000. Incorporation of uridine was assessed by liquid scintillation counting in a Packard Tri-carb counter. For the determination of growth factor production, supernatants were prepared by resuspending the pellet followed by centrifugation to remove the cells. Aliquots of spread and crowded cell supernatants were stored at −20°C until required.

RESULTS

Both PNCM and 50% iron-saturated Lf (Fe-Lf) were able to significantly reduce the incorporation of ^{3}H-UdR into a 72-hour MLC. However, as can be seen from Fig 1, significant inhibition could only be achieved if the cells of the MLC were crowded together in round-bottomed wells of tissue culture plates. When compared with cultures spread in flat-bottomed wells, the crowded cultures resulted in considerably greater stimulation. It was this stimulation, resulting from cell crowding, that was inhibited by both PNCM and Lf.

Figure 2 shows the effect of removal of Lf from PNCM by monoclonal antilactoferrin bound to CNBr-activated Sepharose columns. It can be seen that this procedure almost completely abrogates the inhibitory activity of PNCM. Previous experiments from ^{59}Fe-labeled Lf had shown that Lf could be recovered from the column with glycine HCl buffer pH 2.8. Lf could therefore be recovered from PNCM, and Fig 2 shows that this Lf retained its inhibitory activity against the MLC.

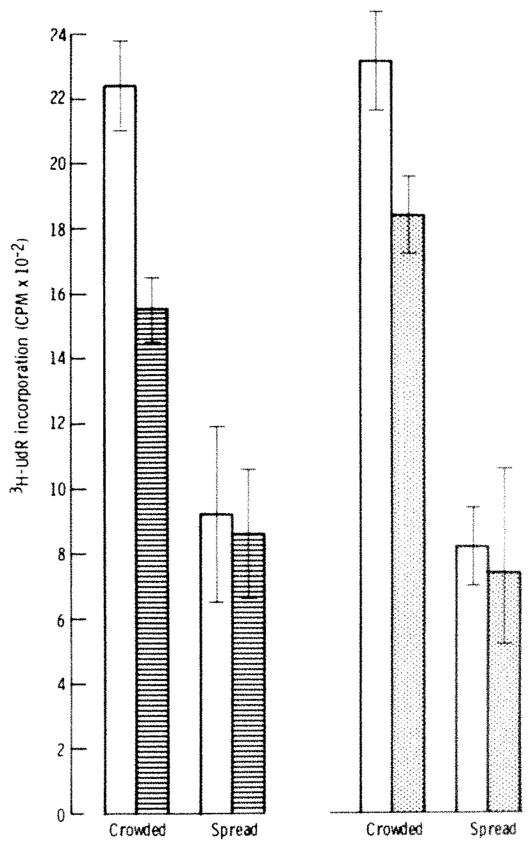

Fig 1. The effect of PNCM and 10^{-10} mol/L Fe-Lf on the MLC in crowded and spread conditions. The results represent the mean of six separate experiments ± SEM using a different PNCM donor for each. □, Control; ▤, + PNCM; ▨, + Fe-Lf.

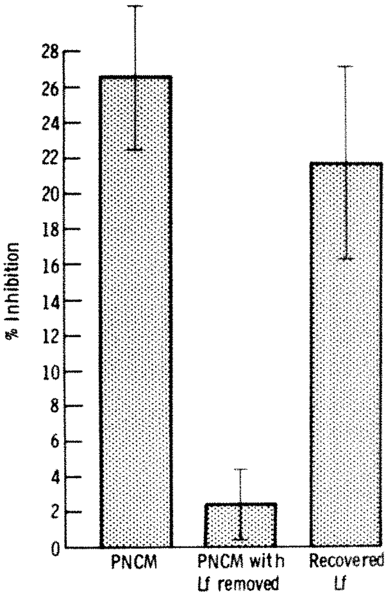

Fig 2. The effect of removal of lactoferrin from PNCM by monoclonal antilactoferrin bound to a CNBr-activated sepharose 4B column. The results show the mean percentage inhibition of ^{3}H-UdR uptake by MLC compared with controls in six separate experiments using a different PNCM donor for each.

Figure 3 shows that polyclonal antilactoferrin in the liquid phase was able to abrogate the inhibitory activity of PNCM. Polyclonal antilactoferrin (Dako-Patts) was extensively dialyzed against PBS at 4°C to remove all sodium azide; serial dilutions of the azide-free antibody were mixed with PNCM and incubated at 37°C for one hour. This procedure resulted in the total abrogation of the inhibitory activity of PNCM.

Figure 4 shows that 50% Fe-Lf inhibits the MLC in crowded cultures at concentrations between 10^{-8} and 10^{-12} mol/L, but the effect is lost at 10^{-14} mol/L. Apo-Lf showed no significant inhibitory activity at any concentration. The dose-response curve for PNCM when expressed in terms of its Lf concentration was the same as purified 50% Fe-Lf. The data illustrated in Fig 4 suggests that the Lf in PNCM, being inhibitory to the MLC, is carrying iron. This was confirmed by the formation of a pink band on the affinity column during the separation of Lf from PNCM, thus indicating that the neutrophil-derived Lf was indeed carrying iron.

Having determined that the inhibitory factor in PNCM was Lf, we then investigated further the effects of Lf on the MLC. As discussed earlier, considerably more stimulation was achieved when the cells of the MLC were crowded together in round-bottomed wells of tissue culture plates than when spread in flat-bottomed wells. It was the extra stimulation induced in the crowded cultures that was inhibitable by Lf. It was therefore assumed that Lf acted on a factor that promotes transcriptional activity and is produced as a result of cell crowding. To test this hypothesis supernatants were collected from 72-hour crowded cultures grown in the presence or absence of PNCM or Fe-Lf. These supernatants were then added to a spread culture with an equivalent volume of cRPMI acting as a control. It can be seen from Fig 5 that crowded culture supernatants significantly increased the stimulation of the spread MLC above that of the control. When supernatants were produced in spread cultures and subsequently transferred to a second spread culture, no increase in stimulation was observed. Thus a factor that increases transcriptional activity was being produced as a result of cell crowding. Figure 5 also shows that both PNCM and Fe-Lf were able to reduce the apparent production of this factor. What is not clear from these data, however, is whether the Fe-Lf is inhibiting the production of the factor or whether carry-over of Fe-Lf in the supernatant is affecting the action of the factor in the spread culture. Consequently Fe-Lf was added after completion of 72 hours crowded culture, and then supernatants were transferred to spread cultures. The results (Fig 6) show significant inhibition of ^{3}H-UdR uptake in the spread culture but less inhibition than expected if carry-over was the whole explanation. Similarly, supernatant from which lactoferrin was removed by passage down an affinity column produced an intermediate level of inhibition (data not shown). Thus Lf appears to have an effect on both the production and the effect of the transcription-promoting factor.

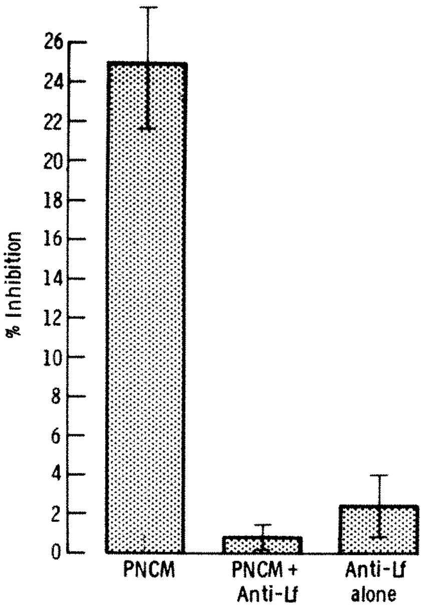

Fig 3. The effect of adding polyclonal antilactoferrin to PNCM. The results show the mean percentage inhibition of ^{3}H-UdR uptake by MLC compared with controls in six separate experiments using a different PNCM donor for each.

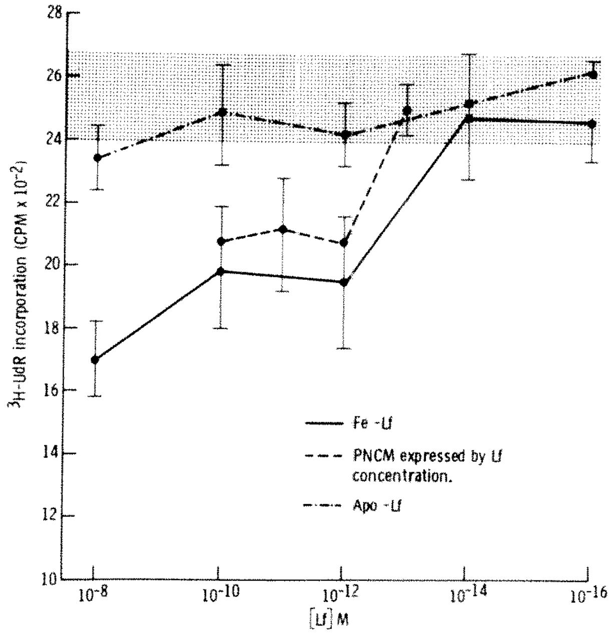

Fig 4. Dose-response curves for apo-Lf, Fe-Lf, and PNCM expressed in terms of its Lf concentration. Shaded area shows the mean and standard error of ^{3}H-UdR uptake by lactoferrin-free control MLCs.

Finally, the time course of Fe-Lf effects was investigated. Figure 7 shows no detectable inhibition by Fe-Lf of ^{3}H-UdR uptake into crowded cultures until 72 hours. When the supernatants from these cultures were transferred to spread cultures the same result was obtained, as only supernatants from 72-hour cultures showed significantly impaired ability to increase ^{3}H-UdR uptake.

DISCUSSION

During phagocytosis of opsonized particles, neutrophils discharge 60% to 70% of secondary granule proteins into the surrounding medium.[5,6] Similarly, neutrophils obtained from

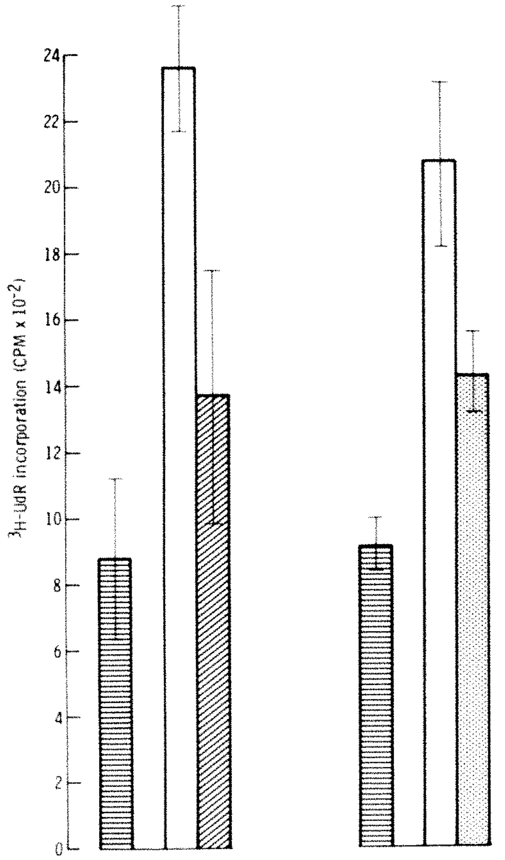

Fig 5. The effect of PNCM and 10^{-10} mol/L Fe-Lf on a transferable transcriptional factor. Supernatants from 72-hour crowded cultures grown in the presence and absence of PNCM or 10^{-10} mol/L Fe-Lf were added to spread cultures and the ^{3}H-UdR uptake compared with a control spread culture in cRPMI alone. Results shown are the mean ± SEM from six separate experiments with a different PNCM donor for each. ▤, RPMI; □, no inhibitor control; ▨, + PNCM; ░, +Fe −Lf.

patients undergoing surgical trauma show evidence of secondary granule discharge with reduced intracellular concentrations of granule proteins accompanied by rising levels in the plasma.[18] Thus there is circumstantial evidence for an extracellular role for secondary granule constituents, including Lf released when neutrophils are activated. At least one possible extracellular action is negative feedback control of lymphocyte responses, which is in keeping with other evidence that Lf inhibits T cell function[12] and antibody production.[9]

The observations reported show that phagocytosing neutrophils release a factor that inhibits uptake of uridine in a MLC. The factor has been clearly identified as Lf, since inhibitory activity was lost when specific antibody was added or when Lf was removed on a monoclonal antibody (MoAb) column and restored when Lf was recovered from the column. The effects of the neutrophil-derived Lf could be reproduced by Lf purified from human breast milk. There was a clear requirement for iron, as the apoprotein lacked activity. This finding was unexpected, as Lf would bind any free iron in the growth medium; the explanation may be

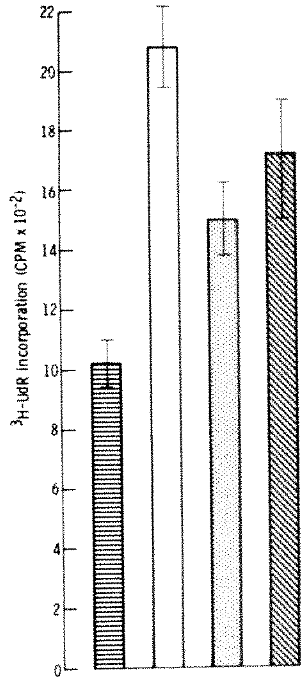

Fig 6. The effect of adding 10^{-10} mol/L Fe-Lf at the beginning and end of a 72-hour crowded culture. Supernatants were then added to spread cultures and ^{3}H-UdR uptake compared with a control spread culture in cRPMI alone. Results shown are the mean ± SEM from five separate experiments. ▤, + RPMI; □, no Lf; ░, Fe −Lf added at initiation of crowded culture; ▧, Fe −Lf added at termination of crowded culture.

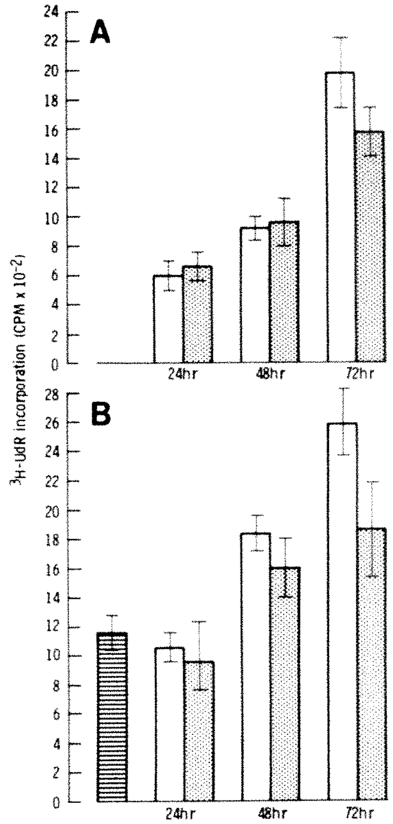

Fig 7. The time course of ^{3}H-UdR uptake by crowded cultures with and without 10^{-10} mol/L Fe-Lf (A). At 24, 48, and 72 hours supernatants from these crowded cultures were transferred to spread cultures and incubated for a further 72 hours (B). The results shown are the mean ± SEM of seven separate experiments. □, No Lf; ░, + Fe −Lf; ▤, + RPMI.

binding of free iron by transferrin from fetal calf serum before Lf was added.

Lf binds to specific receptors identified on mononuclear phagocytes and B lymphocytes[19,20] where it presumably delivers iron that is subsequently involved in reactions limiting the production or action of factors influencing transcription and protein synthesis. There is very little data about the iron saturation of Lf in neutrophil granules, but it is usually considered to be iron free,[1,4] whereas both the pink color when neutrophil-derived Lf is retained on an antibody column and its inhibitory action against the MLC suggest that when released it carries iron. This raises the intriguing possibility that Lf released passively by cell death is iron free and inactive, while Lf released by activation of the neutrophil acquires iron and, with it, inhibitory activity. The sources of iron may be microbial ferritin, as superoxide radical generated by the neutrophils' respiratory burst and involved in microbial killing can certainly mobilize iron from ferritin.[21] During an inflammatory response there must be a balance between factors such as endotoxin and complement breakdown products, providing a positive stimulus to mononuclear cells and negative factors such as Fe-Lf released from neutrophils when invading organisms are phagocytosed and killed. Initially the reaction will be driven, but as it succeeds it must be inhibited.

An argument against a physiologic regulatory function for Lf is the concentration at 10^{-9} mol/L reported in normal plasma,[22,23] as this is well within the concentration range at which Lf affects lymphocyte growth (Fig 4). One explanation may be the failure to distinguish between iron-free inactive molecules and the iron-carrying active form. A more likely explanation is the difficulty in avoiding neutrophil activation and secondary granule discharge during venesection, since when great care is taken the plasma Lf concentration may be only 10^{-13} mol/L and below the concentration effective on lymphocyte responses.[24]

The present study has shown that a neutrophil product, namely Lf, inhibits RNA transcription (^{3}H-UdR uptake) in a MLC. It is possible to show the same, although less marked, effect upon DNA synthesis using labeled thymidine (data not shown). When the cells were crowded in a round-bottomed well, there was considerably more uridine uptake than when spread in a flat-bottomed well, and this effect appeared to be due to a soluble factor, since it was transferable in the supernatant from crowded cultures. The inhibitory action of Lf was on this factor, either reducing its production or its transcription-promoting effect or both. Experiments adding Lf to crowded culture supernatants or removal of Lf from supernatants produced in its presence appeared to show an effect upon both growth factor production and transcription-promoting activity. These results might be explained by inhibition of factor production in both the crowded cultures and spread cultures, as transcription-promoting factors are probably produced as a secondary event in the stimulated spread cultures. Specific assays are being performed to define the factors involved.

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Quantitation of Human In Vitro Megakaryocytopoiesis by Radioimmunoassay

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The isolation and characterization of human megakaryocyte growth factors has been hampered because evaluation of megakaryocyte growth in semisolid medium requires both lengthy incubation and visual quantitation. In addition, colony formation requires cell division, while most regulation of platelet production may involve individual, nonproliferating differentiating megakaryocytes. We have developed a radioimmunoassay (RIA) that makes use of an iodinated murine monoclonal antibody (MoAb) specific for platelet/megakaryocyte glycoprotein IIb/IIIa (GPIIb/IIIa) to measure megakaryocyte production in liquid marrow culture. This assay is sensitive to 3 × 10^3 platelets (roughly 30 megakaryocytes) and linear up to 1 × 10^6 platelets, and thus it provides a useful range for quantitating megakaryocyte production in in vitro marrow culture. Significant differences (threefold to fivefold) in megakaryocyte/platelet-specific GPIIb/IIIa complex are detected between stimulated and unstimulated marrow cultures by day 7, although antigen accrual in stimulated cultures continues through at least day 16. Conditions that promote megakaryocyte growth in semisolid medium (ie, aplastic plasma and PHA-LCM) have also been facilitory in liquid culture. This rapid and sensitive assay for cell-bound GPIIb/IIIa should facilitate recognition and isolation of megakaryocyte and platelet growth factors.

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ACCUMULATING evidence suggests that the regulation of megakaryocyte production in mammals is exerted at at least two different steps in the megakaryocyte-platelet differentiation pathway.[1-3] Increasingly sensitive assays for megakaryocyte colony formation have facilitated the study of the early (proliferative) phase of megakaryocytopoiesis.[4-6] This phase of platelet production is, however, the least sensitive to acute changes in platelet count[1,7-10] and thus arguably less important in day-to-day platelet number homeostasis. Because most of the control of platelet production may be exerted upon single cells that ultimately become multinucleate, expand their membrane, and mature cytoplasmically to produce platelets, it seems appropriate to develop an assay that would be sensitive to megakaryocyte maturation. In addition, colony assays for megakaryocytopoiesis require 11 to 15 days of incubation before colonies (CFU-M) can be accurately counted by visual inspection.[5,6] A less time-consuming and labor-intensive assay for use as a screening test for growth factors was therefore most desirable.

The glycoprotein IIb/IIIa (GPIIb/IIIa) complex is present on all recognizable human megakaryocytes and platelets from normal donors.[11,12] Fluorescent antibodies against this complex have been extremely useful as specific markers for megakaryocytic colonies and in identification of earlier, morphologically unrecognizable megakaryocytic cells from both cultures and patient materials.[4,11-14] As Jenkins et al[15] have shown using biosynthetic labeling of isolated CFU-M-derived human megakaryocytes, the GPIIb/IIIa complex is made by megakaryocytes but is not synthesized in detectable amounts by monocyte/macrophage or erythroid colonies. Thus the cell-bound GPIIb/IIIa in a heterogeneous marrow culture cell population specifically reflects megakaryocytic lineage cells present, whether they are platelets, morphologically recognizable megakaryocytes, or early antigen-bearing small round cells. Similar increases in cell surface membrane GPIIb/IIIa would be observed in cultured marrow when more small cells are present, or when a few individual cells mature and enlarge, or with development of both kinds of cells.

We have developed a radioimmunoassay (RIA) to detect cell-bound GPIIb/IIIa that allows rapid quantitative assessment of in vitro megakaryocytopoiesis. Because the assay is relatively easy to perform, it should be useful in screening for megakaryocyte growth factors. Because accumulation of GPIIb/IIIa is associated with cytoplasmic maturation[11,12] as well as with megakaryocyte proliferation, the assay ought to detect growth factors active in the terminal events of megakaryocytopoiesis or platelet production, in addition to factors that influence megakaryocyte proliferation.

MATERIALS AND METHODS

Marrow culture and cell preparation. Nucleated bone marrow cells were obtained from normal volunteers who had given informed consent consistent with institutional guidelines. Marrows were collected into preservative-free heparin (GIBCO Laboratories, Grand Island, NY), diluted one to three in phosphate-buffered saline (PBS), and spun at 120 g for 15 minutes to remove platelet-rich plasma.[16] Red blood cells (RBCs) and granulocytes were separated from marrow mononuclear cells by density gradient separation (Histopaque-1077, Sigma Diagnostics, St. Louis), and another slow spin was done. Adherent cells and additional platelets were depleted by incubation of mononuclear cells for 60 minutes in 10% fetal calf serum in plastic flasks. Assessment of residual platelet/megakaryocyte lineage cells during marrow preparation using the RIA described below reveals 30% to 40% depletion of antigen by each slow spin. Thus 15% to 20% of starting activity remained before adherance, 5% to 10% more antigen was removed by the adherence step, and plated cells cultured contained about 10% of starting cell-bound GPIIb/IIIa.

Marrow culture was performed in 24-well tissue culture plates (GIBCO), at 37°C, 5% CO$_2$, and humidified atmosphere. Three to 5 × 10^5 nonadherent, light-density bone marrow cells were cultured

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in Iscove's modified Dulbecco's medium (GIBCO) supplemented with 30% serum or plasma, 5×10^{-5} mol/L 2-mercaptoethanol, and growth factors as noted, in 1 mL final volume. Plasma and serum samples were obtained from consenting normal volunteers and were filtered through 0.2-μ filters prior to use in cultures. Plasmas were prepared from blood drawn into syringes containing preservative-free heparin. Aplastic plasma was the gift of H. Messner, Toronto, Ontario, and fetal bovine serum (FBS) was purchased (Hyclone Laboratories, Logan, UT). PHA-LCM was prepared as previously described.[17]

Human umbilical vein endothelial cells cultured to confluence over five days as previously described[18] were kindly provided by J. Hoak. After detachment from flasks by a 0.02% EDTA rinse followed by incubation in 0.5% Trypsin (five minutes, 37°C), endothelial cells were washed, counted, and assayed by RIA as above.

Megakaryocytes were quantitated in individual wells by counting nonadherent cells using a hemacytometer and multiplying by the percentage of recognizable megakaryocytes found on a stained cytocentrifuge preparation (1,500 cell differentials).

Radioimmunoassay HP1-1D is a murine antihuman glycoprotein IIb/IIIa complex monoclonal antibody (MoAb) of IgG_{2B} isotype.[13,15] The antibody is specific for human platelet or megakaryocyte GPIIb/IIIa, and does not recognize cells of other blood or marrow cell lineages.[13-15] IgG was isolated from the ascites by protein A-Sepharose[19] (Sigma) and iodinated using Iodogen-treated tubes (Pierce Chemical Co, Rockford, IL) and Iodine-125 (^{125}I; New England Nuclear, Boston). Platelets were isolated using the Mustard technique[16] or were obtained in lyophilized form (Bio/Data, Hatboro, PA). The binding of ^{125}I HP1-1D to platelets and cultured marrow was examined in phosphate-buffered saline (PBS) containing 20% bovine calf serum to block nonspecific and F_c binding and 0.002% EDTA to prevent clumping. After 30 to 60 minutes of incubation, binding was complete at 4°C and at 22°C, and subsequent RIAs were routinely performed at 4°C with one-hour incubation. On several occasions, however, we did observe small (<15% of total counts bound) increases in ^{125}I-HP1-1D bound between 60 and 180 minutes both to fresh platelets and to cultured marrow. This may have been due to slow access of antibody to antigen in the canalicular system or to possible antigen exposure due to platelet swelling in EDTA. No slow, late increase in binding was observed when marrow cultured 12 days in FCS alone was assayed (the differential included 20% macrophages, <0.1% megakaryocytes). It is possible that by choosing one-hour incubations we did not always maximize binding to platelet lineage cells; however, all tubes for a given assay were stopped within a minute or two of each other (by dilution with cold carrier cells and centrifugation) so that comparisons of binding within sets of tubes are valid.

For the routine assay, bone marrow cells or platelets were suspended in assay buffer (20% heat-inactivated bovine calf serum, 0.002% EDTA in PBS), pelleted, and supernate decanted. The pellet was resuspended in 100 μL chilled assay buffer and 100 μL of iodinated HP1-1D in chilled 0.1 mol/L sodium phosphate buffer, pH 7.4; 1% FBS was added (usually 400,000 cpm or approximately 0.02 μg antibody). The mixture was incubated at 4°C with intermittent shaking for 60 minutes, following which 800 μL chilled buffer containing 4×10^6 GPIIb/IIIa-negative carrier cells (usually Jurkat T-lymphoblastoid cells) were added and mixed. After pelleting, the supernate was aspirated, the cells washed once in 1-mL assay buffer, and the pellets counted with a gamma counter. For competition assays, cold antibody was incubated with target cells for 30 minutes at 4°C prior to addition of ^{125}I-HP1-1D. Control MoAbs included anti-TAC, which is specific for interleukin 2 (IL2) receptors (the gift of T. Waldmann), and α-PS, which is specific for the coagulation protein S (the gift of W. Church). Assays from marrow cultures were done in duplicate or triplicate on 200-μL aliquots of resuspended cells from each well.

Autoradiography. Cells for autoradiography were labeled with ^{125}I-HP1-1D, as described in the section above, but subjected to one additional wash. Suspended samples were then centrifuged onto clean glass slides by cytocentrifuge, dipped in photographic emulsion (NTB-2, Kodak, Rochester, NY), and developed for approximately two days at 4°C. Following development with D-19 (Kodak), the slides were equilibrated with buffer and stained with Wright's stain.

RESULTS

The RIA, as developed using iodinated HP1-1D, can detect as few as 3×10^3 platelets and is linear between 10^4 and 10^6 platelets (Figs 1 and 2). This latter level coincides with calculated antigen excess, if one assumes 40,000 glycoprotein IIb/IIIa complexes per platelet.[20] Four separate antibody iodinations and over 50 platelet titrations have yielded completely reproducible results. Approximately 0.02 μg of antibody and 400,000 counts per minute are added to each assay tube. If the average megakaryocyte has approximately 100 times the surface area of a platelet,*[22] this assay can detect accurately between 100 and 10,000 megakaryocytes per aliquot.

The specificity of iodinated HP1-1D binding to platelets was ascertained by competition experiments using cold HP1-1D, a murine IgG monoclonal nonreactive with platelets (anti-TAC), and murine polyclonal IgG (Fig 2). Binding of labeled antibody was competitively blocked by increasing concentrations of cold HP1-1D but not by either of the nonspecific antibody preparations. Competition experiments using cultured (day 14) marrow cells similarly demonstrated blocking by cold HP1-1D but not by another murine monoclonal of the same isotype (α-PS is specific for the coagulation protein S and does react with human platelets; Table 1).

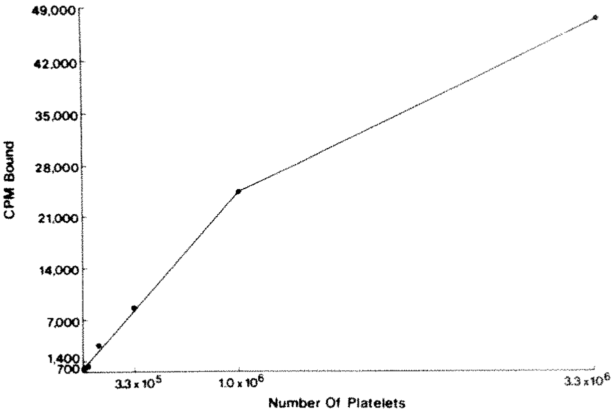

Fig 1. Binding of ^{125}I-HP1-1D to fresh human platelets. Data shown are the mean of triplicates. Very similar curves were obtained using platelets from different donors or commercially obtained lyophilized platelets.

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*The formula for the surface area of a sphere is πd^2. Given an average platelet diameter of 3 μ, and an average megakaryocyte diameter of 30 μ,[21] a ratio of platelet surface area to megakaryocyte surface area of 100 can be calculated.

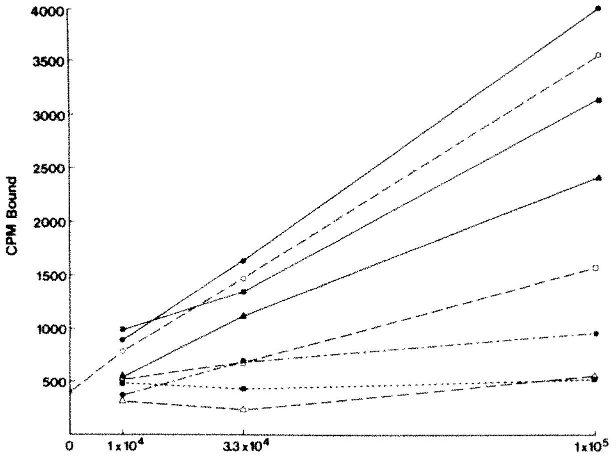

Fig 2. Binding of ^{125}I-HP1-1D to platelets (O——O) is not influenced by pre-incubation of platelets with nonspecific antibodies (1.0 μg α-TAC, ●——●; 1.0 μg murine γ globulin, ■——■). Increasing amounts of cold HP1-1D progressively inhibit binding of the iodinated antibody (0.0075 μg HP1-1D, ▲——▲; 0.015 μg HP1-1D, □----□; 0.03 μg HP1-1D, ●–●–●; 0.06 μg HP1-1D, ■---■; 0.12 μg HP1-1D, △---△).

Iodinated HP1-1D did not bind to cultured human umbilical vein endothelial cells, with counts bound in a titration from 10^4 to 10^6 cells being equivalent to that of the Jurkat T-lymphoblastoid control (<500 cpm). To be sure that trypsin and EDTA used to remove adherent endothelial cells from flasks before the RIA had not destroyed endothelial cell GPIIb/IIIa, platelets were subjected to similar treatment. EDTA-trypsin treatment of platelets caused only a 20% to 30% decrease in binding of ^{125}I-HP1-1D.

Human light density nonadherent bone marrow cells grow well in liquid culture, with formation of recognizable erythroblasts, macrophages, granulocytes, and megakaryocytes depending on the growth conditions. Using the RIA we monitored total cell-bound GPIIb/IIIa present on marrow cells cultured over 15 days in various conditions. The results of a representative experiment are depicted in Fig 3 and Table 2 gives summary data. During the first four to seven days in culture, cell-bound GPIIb/IIIa falls, presumably as residual platelets in the marrow preparation disintegrate, adhere to the plates, or are ingested by other cells. Culture of marrow in media supplemented with fetal bovine serum (FBS) by itself permitted minimal antigen accrual over 2 weeks time. However, FBS reproducibly supported a response to PHA-LCM that was usually significant by day 7 in culture and always two to four times that of FBS alone by day 14. Aplastic human plasma routinely supported the formation of cell-bound glycoprotein IIb/IIIa complex by day 7. Antigen accrual continued through day 15 and was markedly stimulated by the addition of PHA-LCM. Thus although the assay detects a significant difference between marrow cultured in FBS alone and that cultured in aplastic plasma with PHA-LCM by day 7 (mean of fourfold difference), by day 14 this difference is far larger (mean of 30-fold, Table 2).

Table 1. Binding of ^{125}I-HP1-1D to Cultured Bone Marrow Cells is Blocked by Cold HP1-1D but not by Another Murine IgG$_{2B}$ Monoclonal, α-PS

Competing Antibody	CPM Bound ± SEM
None	4221 ± 83.4
HP1-1D (1 μg)	612 ± 55.2
α-PS (1 μg)	4559.5 ± 73.9

Target cells were aliquots of marrow cells (10^5 cells/aliquot) that had been cultured 14 days in 30% FCS + 5% PHA-LCM. Cells were reacted 30 minutes at 22°C with competing antibody prior to addition of ^{125}I-HP1-1D.

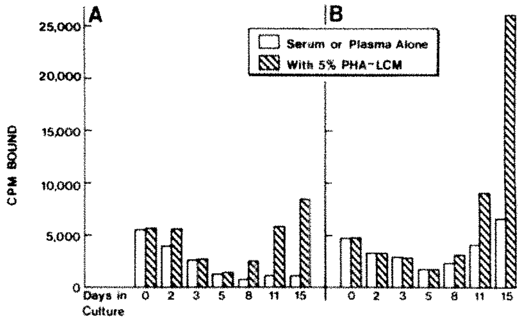

Fig 3. Megakaryocytopoiesis in liquid cultures supplemented by (A) fetal bovine serum or (B) aplastic human plasma, as measured by cell-bound ^{125}I-HP1-1D. Five hundred thousand marrow cells per well were cultured with or without PHA-LCM for varied numbers of days in this representative experiment.

We found that bone marrow cells cultured with 30% normal human plasma or serum had little cell-bound glycoprotein IIb/IIIa after two weeks in culture (Fig 4). The addition of PHA-LCM to these cultures had variable effects, with some normal samples allowing an increment of twofold or threefold antigen increase over two weeks time, but with the majority of normal plasmas and sera not supporting such megakaryocyte growth (Fig 4). For a given donor, plasma and serum usually had similar growth supporting characteristics (the plasma and serum samples in Fig 4 are from the same donors).

The RIA proved very reliable in measuring megakaryocyte lineage cells in liquid culture in over 40 experiments using different bone marrow samples. Microscopic examination of cytospins from the cultures after day 5 revealed recognizable megakaryocytes proportionate to bound counts (Fig 5). The standard error between aliquots from a given well (duplicates or triplicates) ranged from 0% to 10% of the mean counts bound and was usually less than 5%. Well-to-well variation (ie, duplicate wells set up on day 1 and compared on the day of assay) was occasionally somewhat greater, but statistically significant differences between unstimulated and stimulated wells (for instance fetal bovine serum with and without PHA-LCM) were present by day 11 even in the most unresponsive marrow. Radioimmunoassays were routinely performed only on easily resuspendable cells; however, when adherent cells were removed with a rubber policeman and assayed, no counts above background were observed.

Visualization of the populations of cells binding iodinated HP1-1D was performed using autoradiography on the cultured marrow populations (Fig 6). Activity bound to recog-

Table 2. Compilation of GPIIb/IIIa Radioimmunoassay Results From Marrow Cultured 7, 10, or 14 Days Under Different Conditions

Culture Conditions	Days in Culture		
	7	10	14
FBS alone	100 (9)	74.5 ± 10.3 (6)	68 ± 7.6 (4)
FBS + PHA-LCM	184 ± 14.7 (9)	489 ± 121 (6)	490 ± 134 (4)
Aplastic plasma	272 ± 35 (9)	640 ± 136 (9)	1106 ± 169 (7)
Aplastic plasma + PHA-LCM	400 ± 58.3 (8)	1095 ± 155.8 (8)	2318 ± 358 (6)

Results for each marrow were expressed relative to the counts bound by cells cultured seven days in FBS alone for that marrow prior to averaging. For each mean percentage, the standard error of the mean is given, with "n" in parentheses.

nizable megakaryocytes in all stages of maturation and, in addition, was found on rare, small round cells similar to those found in megakaryocyte colonies.[5,12] Activity was not found on monocytes, macrophages, granulocytes, or erythroblasts.

DISCUSSION

We have developed an accurate, specific, and rapid screening RIA for cell-bound GPIIb/IIIa that is useful for the quantitation of human megakaryocytes grown in liquid marrow culture. GPIIb/IIIa on the surface of cells is quantitated by use of an iodinated MoAb specific for the human platelet or megakaryocyte GPIIb/IIIa complex. Bound antibody is separated from free antibody by centrifugation of cells and the pellet counted in a gamma counter.

The presence of the GPIIb/IIIa complex on megakaryocytic lineage cells ranging from small round morphologically undifferentiated cells to recognizable mature megakaryocytes and platelets has been well described[11,12] and the complex has been used by many investigators as a target for immunofluorescent probes in characterizing cells from colonies.[4,11-13,23] Some investigators have questioned the specificity of GPIIb/IIIa to the megakaryocytic lineage, however, as similar proteins cross-reactive with certain antibodies made against GPIIb/IIIa or GPIIIa have been described in monocyte, endothelial, smooth muscle and fibroblast-like cell preparations.[24-28] It is likely that shared antigens in the α-chain of a family of similar membrane glycoprotein complexes explain this cross-reactivity.[28]

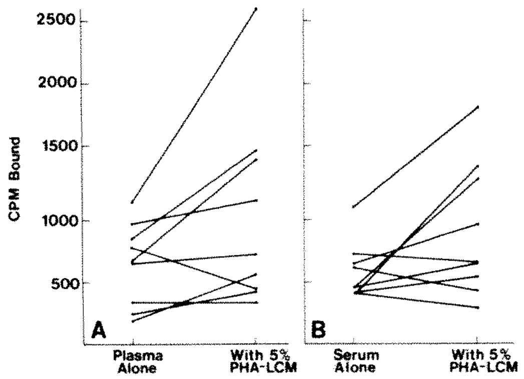

Fig 4. Megakaryocytopoiesis in liquid cultures supplemented by normal human plasma or serum as measured by cell-bound ^{125}I-HP1-1D. Three hundred thousand marrow cells per well were cultured with or without added PHA-LCM two weeks prior to assay. (A), Normal human plasma; (B), normal human serum.

HP1-1D antibody appears to be specific for the GPIIb/IIIa complex rather than for one of the two glycoproteins, as dissociation of the complex by incubation of platelets with EDTA at 37°C[29] reduces HP1-1D binding to platelets, and the antibody does not specifically bind to electrophoretically separated GPIIb and GPIIIa in Western blots (Nichols, unpublished data). We have not found cross-reactivity of HP1-1D with antigens or cells from outside the human megakaryocyte/platelet lineage.[13,15,22] In the present study we examined cell types found in marrow cultures and found no evidence that ^{125}I-HP1-1D bound to monocytes, macrophages, endothelial cells, or fibroblasts (by RIA, autoradiography, or both). We have previously reported the biosynthesis of GPIIb/IIIa by megakaryocyte colonies (CFU-M) plucked from methylcellulose marrow cultures.[15] In those studies our monoclonal against the human GPIIb/IIIa complex, HP1-1D, was used to purify the ^{35}S-methionine-labeled complex from cell lysates, and no detectable GPIIb/IIIa was synthesized by monocyte/macrophage colonies. Thus to the best of our knowledge HP1-1D antibody is specific to the cells of interest in this investigation.

The sensitivity of the RIA is probably similar to that of colony assays, but since very different products are measured in the different assays, this comparison must be only a general one. Investigators report megakaryocyte colony sizes ranging from three to 500 cells, with mean colony size less

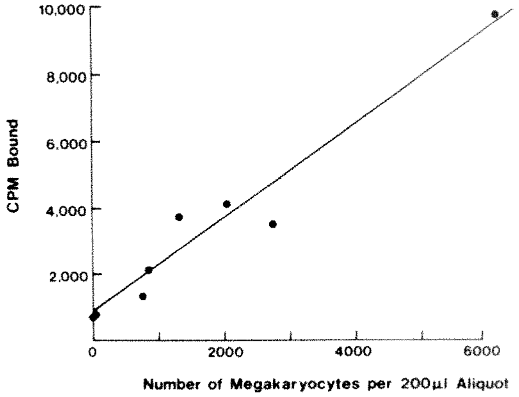

Fig 5. Counts bound are proportionate to recognizable megakaryocytes after 12 days in various liquid culture conditions (r = 0.97). Megakaryocytes per fraction were determined by multiplying the number of cells per aliquot (as determined by hemacytometer count) by the percentage of megakaryocytes in a differential of 1,500 cells done on a cytospin from that well. Percentages varied in this representative experiment from 0% to 3%.

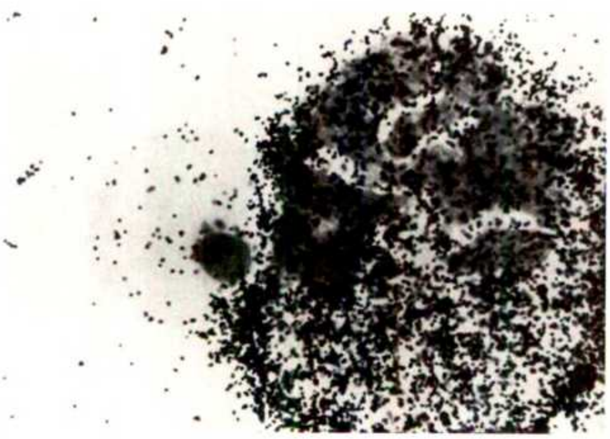

Fig 6. Autoradiographic demonstration of the specificity of ^{125}I-HP1-1D binding to cells. Many grains are seen over the Stage III megakaryocyte, while few are seen over an adjacent macrophage. (Original magnification × 1,000, current magnification × 680.)

than 100 cells.[4-6] No colonies are routinely formed in unstimulated (normal human plasma) cultures, whereas aplastic plasma and PHA-LCM permit up to 50 colonies per 10^5 marrow cells.[4-6,23] In unstimulated liquid cultures we find 500 to 1,200 CPM bound, which corresponds to about 1 to 2 × 10^4 platelets or 10 to 20 megakaryocytes. Mature megakaryocytes are found on cytospin preparations of these cultures, implying that some individual cells may mature in vitro to megakaryocytes under conditions where no colonies are formed. Marrow cultured in aplastic plasma and PHA-LCM binds 4,000 to 30,000 CPM per 10^5 starting cells by day 14, which by extrapolation from platelet-binding curves corresponds to 1,000 to 10,000 megakaryocytes. We stress that although these calculations demonstrate that colony assays and liquid marrow culture assayed by RIA detect about the same amount of megakaryocyte growth from human marrow preparations, the product measured is very different. The unit of measure in a colony assay is the colony that represents proliferative and differentiative changes occurring among the progeny of one stem cell over time in culture. On the other hand, in the RIA the unit of measure is a GPIIb/IIIa complex expressed on the surface of a cell where it can bind radiolabeled antibody. Cells binding radiolabeled antibody may have differentiated in culture, proliferated in culture, or both.

The GPIIb/IIIa RIA for evaluation of megakaryocyte growth in culture is faster than semisolid culture assays in three respects. The first two respects are technical and involve the time it takes to set up the cultures and to perform the assays. Liquid marrow cultures may be set up much more quickly than semisolid cultures because they require only the addition of measured volumes of media, cells, and variables to 24-well plates. No suspension in methylcellulose, plasma clot or agar, or preparation of individual culture dishes is required. The time for quantitation is much reduced by the RIA as well. We routinely set up 50 to 75 different growth conditions on a given marrow and assay them all in duplicate or triplicate in about four hours. Counting colonies using an inverted microscope or an immunofluorescent microscope takes approximately five to 15 minutes per plate, so that quantitation of the amount of growth for a given condition in triplicate might take 20 to 40 minutes and that for 50 different conditions several days.

The third-time advantage the RIA has over semisolid marrow culture is that it is sensitive to the presence of growth factors after relatively short incubation times. Megakaryocyte colonies under maximally stimulated conditions (aplastic plasma and PHA-LCM) are recognizable by day 8 or 9, with more colonies formed or becoming recognizable through day 14 to 16. Statistically significant differences between stimulated and unstimulated liquid culture wells are detected by the RIA as soon as days 5 to 7, although in liquid culture also antigen accrual continues through day 15.

The formation of megakaryocyte colonies in semisolid medium by definition requires that the committed cells undergo cell division, while much of the regulation of megakaryocyte maturation and platelet shedding probably occurs on cells that undergo few if any further cell divisions. Thus, colony assays, while sensitive for factors that promote or permit proliferation (colony stimulating factors), may not detect regulators of megakaryocyte maturation (megakaryocyte maturation factors). Because this GPIIb/IIIa RIA of liquid marrow culture can measure increases in cell membrane in addition to increases in cell number, we hope that it will be especially useful in recognition and isolation of megakaryocyte maturation factors.

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Production of Colony-Stimulating Activity by Normal and Neoplastic Human B Lymphocytes

By V. Pistoia, R. Ghio, S. Roncella, F. Cozzolino, S. Zupo, and M. Ferrarini

Normal human B cells were purified from peripheral blood or tonsils and tested for their ability to release colony-stimulating activity (CSA) in short-term cultures. The target cells used in the CSA assays were from peripheral blood or bone marrow. Unstimulated B cells produced CSA in amounts similar to those present in the GCT-conditioned medium used as a positive control. The B cell-derived CSA predominantly promoted the growth of colonies that contained macrophages alone or macrophages and granulocytes. CSA eluted in a single peak from a G-75 Sephadex column with an approximate molecular weight (mw) of 65 to 70 kilodaltons (kd). Fractionation of tonsil B lymphocytes on Percoll density gradients showed that large B cells, probably already activated in vivo, were the main source of CSA. By contrast, small, resting B cells recovered from a different fraction of the Percoll gradient released minimum amounts or no CSA. However, these B cells became CSA producers following stimulation with *Staphylococcus aureus* Cowan (SAC) in vitro. B cells purified from the peripheral blood of nine out of 12 patients with B-cell chronic lymphocytic leukemia (B-CLL) also released CSA in vitro in the absence of stimuli. These findings suggest that by releasing CSA, B cells may have a role in the regulation of hematopoiesis and in the control of the inflammatory process.

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COLONY-STIMULATING FACTORS (CSFs) are glycoproteins that promote the in vitro growth of granulocyte and monocyte/macrophage colonies from committed progenitors.[1] CSFs are believed to have a fundamental role in stimulating hematopoiesis in vivo. Besides promoting cell growth and differentiation, CSFs may also participate in the induction of the inflammatory reaction and in the potentiation of a number of effector mechanisms.[2-7]

Two main types of human CSF have been identified, namely α-CSF, which induces neutrophil, monocyte/macrophage, and eosinophil colony formation; and β-CSF, which selectively stimulates neutrophil colonies.[1] An additional type of human CSF, active only on cells of the monocyte/macrophage lineage (CSF-1), has been characterized and cloned.[8,9]

Numerous cell types produce factors with colony-stimulating activity (CSA) some of which are not yet completely characterized. These cells include T cells,[10,11] monocytes/macrophages,[12,13] endothelial cells,[14,15] and fibroblasts.[16]

This study demonstrates that normal B cells also release CSA in vitro and that CSA production is mainly a property of activated B cells. These data suggest that, when activated, B cells may take part in the inflammatory response and in the regulation of hematopoiesis. Normal B cells and malignant B lymphocytes from a number of CLL patients were found to release CSA.

MATERIALS AND METHODS

Cell fractionation procedures. Peripheral blood mononuclear cells (MNC) from normal donors were separated on Ficoll-Hypaque (F-H) density gradients. Monocytes were removed partially by adherence. T cells were isolated from the suspensions by rosetting with neuraminidase-treated sheep erythrocytes (E), as previously reported,[17] followed by fractionation on two subsequent F-H density gradients. Indicator cells were removed from E rosetting cells by hypotonic lysis.[17] When indicated, T cells were further depleted of monocytes by treatment with an anti-HLA-DR monoclonal antibody (PTF 29/12) and complement. B cells were purified as previously described.[18]

Briefly, non-T cells (E rosette-negative cells) were depleted of monocytes by treatment with the OKM1 monoclonal antibody (Ortho Pharmaceutical, Raritan, NJ) and complement. They were subsequently sensitized with the B1 monoclonal antibody (Coulter, Hialeah, FL). Then 3×10^7 sensitized cells were rosetted with 2 mL of 5% ox erythrocytes coated with a purified rabbit antimouse Ig antibody. Rosetting cells ($B1^+$ cells) were separated from nonrosetting cells ($B1^-$ cells) on F-H density gradients. Adherent monocytes were recovered from plastic with a rubber policeman. Suspensions of tonsil lymphocytes were prepared by gentle mincing. Separation of MNC on F-H density gradients and depletion of E rosetting cells was carried out as above. Non-T cells were subsequently treated with the OKM1 antibody and complement. In order to separate large B cells from small B cells, tonsil non-T cells were fractionated on a Percoll density gradient.[17] Cells (5×10^7) were resuspended in 2 mL of 100% Percoll (Pharmacia, Uppsala, Sweden) solution in a 15-mL conical test tube. Four additional Percoll concentrations (diluted in medium supplemented with 10% FCS) of 60, 50, 40, and 30% were layered over the 100% fraction starting from the bottom of the tube.

Cells were spun at 3,000 rpm for 15 min. Four cell fractions were obtained: the first fraction contained low density cells, consisting predominantly of large B cells; the fourth was enriched for small B cells. The second and third fractions comprised cells of intermediate characteristics.

Cells from the first and fourth fractions only were washed twice and used for further tests. Cells from twelve B-CLL patients were also studied. Peripheral blood samples from B-CLL patients were obtained after informed consent. The diagnosis of B-CLL was established by clinical, morphologic, and immunological criteria. MNC suspensions were depleted of cells forming E rosettes, as

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above, and treated with the OKM1 monoclonal antibody and complement.

Cell surface marker analysis. Cells with receptors for sheep erythrocytes or surface immunoglobulin (sIg) were detected as previously reported.[17] The murine monoclonal antibodies used in this study were the pan-T reagent, OKT3 (Ortho Pharmaceutical); the B cell specific antibody, B1 (Coulter Electronics); the natural killer (NK) cell specific reagent, Leu 11 (Becton Dickinson, Sunnyvale, CA); and the anti–monocyte-macrophage, OKM1 (Ortho Pharmaceutical), which also stains NK cells. The anti-Tac monoclonal antibody that identifies IL-2 receptors[19] was kindly provided by Dr T. Waldman. The anti–HLA-DR monoclonal PTF 29/12[20] was kindly donated by Dr G. Damiani. All of the above reagents were used in indirect immunofluorescence with a fluorescein-isothiocyanate (FITC)-conjugated goat F(ab')$_2$ antimouse immunoglobulin. Wet cell preparations were examined with a Leitz-Orthoplan fluorescence microscope. The percent of positive cells was calculated on at least 200 cells per preparation.

Cell cultures. Cell suspensions enriched for T cells, monocytes, or B cells were cultured for 48 hours at the concentration of 1×10^6/mL in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco) (RPMI-FCS) or, alternatively, with 2% endotoxin-free human albumin (ISM, Milano, Italy).

In some experiments, small resting B cells were cultured for 48 hours at a concentration of 1×10^6 in RPMI-FCS, with killed *Staphylococcus aureus* of the I Cowan strain (SAC), kindly donated by Dr S. Romagnani.[17] In some experiments, B-cell supernatants were absorbed onto Concanavalin A (Con A) bound to Sepharose beads (Sepharose-Con A) (Pharmacia, Uppsala, Sweden) or on human albumin coupled to Sepharose beads. Three mL of supernatants were admixed with 150 μL of Sepharose beads and incubated for six hours at room temperature in a rotating mixer apparatus. Beads were subsequently removed by centrifugation. All of the supernatants from the above cultures were harvested following centrifugation, filtered, and stored at −20°C until tested.

CSA assay. The 1×10^5 bone marrow cells or 2×10^5 peripheral blood MNC, isolated on F-H density gradients and depleted of both adherent cells and E rosetting cells, as above, were used as targets for the assay. Cells were resuspended in a final volume of 1 mL of a mixture containing α-medium (Gibco) 10% heat-inactivated FCS and 0.3% agar and supplemented with non-essential amino acids, L-glutamine, and sodium pyruvate.[21] Test supernatants were included in the culture medium at the final concentration of 10%. Control plates contained the conditioned medium from the GCT cell line[22] (Gibco) as a source of CSA. Cultures were incubated in 95% air and 5% CO$_2$ at 37°C and scored for colonies after 14 days. On a few occasions, seven-day colonies formed by bone marrow cell suspensions were also counted. A colony was defined as an aggregate of more than 50 cells.

In some experiments, B-cell supernatants were tested for the ability to support myeloid colony formation by murine bone marrow cells (from the Balb/c mouse strain) collected from individual femoral shafts by flushing the marrow cavity. Cells were subsequently isolated on F-H density gradients, washed, and cultured in agar.

Cell types within colonies were identified by morphological and cytochemical criteria. Each dish was washed twice, with Hanks' balanced salt solution (HBSS). Agar was subsequently removed by floating in distilled water and was dried onto glass slides under filter paper.[23] Dried cultures were fixed with acetone-methanol fixative (60% acetone, 10% methanol, 30 mmol/L sodium citrate, pH 7.4) and stained with Giemsa for morphological examination. Slides were also stained for napthol-ASD-chloroacetate esterase to detect cells of the granulocyte lineage,[24] and for alpha-naphthyl-acid esterase to identify monocytes/macrophages.[25] Eosinophil colonies were recognized by bright intracytoplasmic green staining with Luxol fast blue (LFB).[25] All of the cytochemical staining was carried out according to published methods.[24,25]

Molecular weight determination of the B cell-derived CSA. (NH$_4$)$_2$SO$_4$ was added to 80 mL of normal tonsil B cell culture supernatant at a final concentration of 75%. The precipitate was centrifuged, resuspended in phosphate-buffered saline (PBS) containing 50 μg/mL gentamicin, and was dialyzed extensively against the same buffer. Aliquots of 3 mL were then filtered on a 2.6-×-100 Sephadex G-75 Superfine column (Pharmacia, Uppsala, Sweden) equilibrated with PBS and calibrated with the following markers: aldolase (158,000 mw), human serum albumin (69,000 mw), Ig light chains (23,000 mw), and cytochrome c (12,300 mw). A flow rate of 10 mL/hr was used, and 2-mL fractions were collected. Aliquots of the fractions were concentrated, filtered on a 0.22-μm filter, and assayed for CSA.

RESULTS

CSA production by normal B cells. Normal B cells purified from peripheral blood or tonsils were cultured for 48 hours. The supernatants were subsequently tested for CSA and found to contain CSA in amounts comparable to those of the GCT-conditioned medium used as a positive control (Table 1). These supernatants were active on both bone marrow and peripheral blood target cells (Table 1). Since peripheral blood represented a more convenient source of hemopoietic precursors, most of the subsequent experiments were carried out on peripheral blood cells.

B cells released CSA in the absence of any apparent stimulus. However, culture media were supplemented with FCS and the possibility existed that endotoxin present as contaminant of FCS could provide the appropriate stimulus. This possibility was made unlikely by the following observations: (a) the batch of FCS used had a very low endotoxin content, as assessed by the Limulus assay; and (b) B cells cultured in medium containing 2% endotoxin-free human serum albumin in the place of FCS retained the ability of producing CSA (Table 1).

A final possibility was that B cells were activated to release CSA by the separation procedure that was making use of the B1 monoclonal antibody. However, (a) both positively (B1$^+$) and negatively (E$^-$OKM1$^-$) selected B cells could produce CSA in culture, and (b) in control experiments, no proliferation (as assessed by ^{3}H-thymidine incorporation) was detected when B cells were stimulated with the B1 monoclonal antibody.

Colonies formed by peripheral blood precursors after 14 days in culture were examined by both Giemsa and cytochemical staining, with approximately 70% to 80% being formed exclusively by monocytes/macrophages. The remaining colonies were of the mixed type and contained both neutrophils and macrophages (15% to 20%), while eosinophilic colonies represented a minor component (5% to 10%). Comparable results were obtained when B cell supernatants were tested on bone marrow cell suspensions and seven-day colonies were examined, with the exception that the number of mixed colonies was higher (50%). In parallel experiments B-cell culture supernatants were tested on both

Table 1. CSF Activity in B Cell Culture Supernatants

Supernatants	Colonies Formed by Peripheral Blood Cells (2×10^5) Exp. 1†‡§	Exp. 2†‡§	Exp. 3†‡‖	Colonies Formed by Bone Marrow Cells (1×10^5) Exp. 4†‡§	Exp. 5†‡‖
Peripheral blood B cells*	23 ± 2	31 ± 4	19 ± 3	125 ± 10	69 ± 5
Tonsil B cells*	19 ± 2	27 ± 2	24 ± 2	133 ± 12	73 ± 3
GCT cell line*	26 ± 3	30 ± 3	29 ± 3	150 ± 15	82 ± 7
Medium (control)	0	3 ± 1	2 ± 1	12 ± 2	7 ± 1

*Tested at a final concentration of 10%.

†Results are mean values ± SE from triplicate cultures.

‡Colonies were counted after 14 days.

§CSA produced by 1×10^6 cells cultured for 48 hours in RPMI-FCS.

‖CSA produced by 1×10^6 cells cultured for 48 hours in RPMI supplemented with 2% human serum albumin.

human and murine bone marrow cells. A higher number of colonies was consistently obtained in the human assay, as assessed by counting the colonies at both seven and 14 days (data not shown).

The purity of the B-cell suspensions isolated from peripheral blood or tonsils was tested by surface marker analysis. These suspensions were comprised almost exclusively of B cells, as shown in Table 2, with minor contaminants of monocytes or of T cells.

Additional control experiments were carried out to determine whether contaminant T cells or monocytes were responsible for CSA production. Purified T cells and monocytes were cultured alone or in combination, with or without phytohemagglutinin (PHA), and their supernatants were subsequently tested for CSA (Fig 1).

Unstimulated T cells, cultured with or without monocytes, did not release CSA (Fig 1A). When stimulated with PHA, the mixture of T cells and monocytes as well as the T cells alone produced CSA (Fig 1B). However, treatment of purified T cells with an anti–HLA-DR monoclonal antibody and complement, which eliminated the few contaminant monocytes, virtually abrogated CSA production (Fig 1B). Monocytes alone never released CSA (Figs 1A and 1B). These findings indicate that under the experimental conditions used T cells and monocytes had to interact in order to release CSA; furthermore, they had to be stimulated with PHA. Taken together, all of these data argue against the possibility that in unstimulated, purified B-cell suspensions CSA was produced by contaminant monocytes or T cells.

Table 2. Surface Markers of Purified B Cells

Surface Marker	% Positive Cells* Peripheral Blood†	Tonsil‡	B-CLL‡
sIg	95 ± 4	97 ± 4	ND§
B1	96 ± 3‖	97 ± 5	95 ± 3
HLA-DR	98 ± 2	96 ± 4	96 ± 4
OKT3	<1	<1	<1
OKM1	<1	<1	<1
Leu 11	<1	<1	<1

*Mean ± SE from three different experiments.

†B cells were isolated from peripheral blood MNC by indirect rosetting according to the expression of B1 antigen (see Materials and Methods).

‡Normal tonsil B cells and B-CLL B cells were obtained by depletion of E rosetting cells followed by treatment with the OKM1 monoclonal antibody and complement (see Materials and Methods).

§Not done.

‖Detected by rosetting during the separation procedure.

Supernatants from B cells purified from two tonsil cell suspensions were ammonium sulphate–precipitated and gel-filtered over a G-75 column. CSA eluted in a single peak with an apparent mw of 65 to 70 kd (Fig 2).

Aliquots of the same supernatants were absorbed onto Sepharose-bound Con A or Sepharose-bound human albumin (control). Treatment with Con-A reduced the capacity of B-cell supernatants to support myeloid colony formation by 50% to 60%, whereas treatment with albumin caused a 10% reduction of colony growth. These findings suggest the glycoprotein nature of the B-cell derived substance(s) responsible for CSA.

CSA is released predominantly by activated B cells. The experiments reported above could be interpreted as indicating that unstimulated B cells release CSA spontaneously. The alternative hypothesis was that the B cell suspensions used contained a number of B cells already activated in vivo. These cells could continue to release CSA once placed in culture. These possibilities were investigated by fractionating tonsil B cells on a Percoll density gradient. Of the four cell fractions so obtained, only the first and fourth were used for further experiments.

Low-density B cells from the first fraction were large and were enriched for activated cells, as shown by the proportion of cells that expressed IL-2 receptors detected by the anti-Tac monoclonal antibody[1,9] (23 ± Tac^+ cells, mean ± SE from three experiments). Furthermore, the 24-hour culture supernatants of these B cells consistently contained high amounts of Ig (1 to 10 $\mu g/10^6$ cells as detected by an enzyme-linked immunoabsorbent assay). These Ig were synthesized and secreted in the apparent absence of stimuli.

In contrast, high-density B cells from the fourth fraction were small, did not express IL-2 receptors, and failed to produce Ig in short-term cultures. These data indicate the resting nature of this particular cell fraction, as opposed to the activated state of large B cells.

Low-density and high-density B cells were compared for their ability to produce CSA in vitro in the absence of

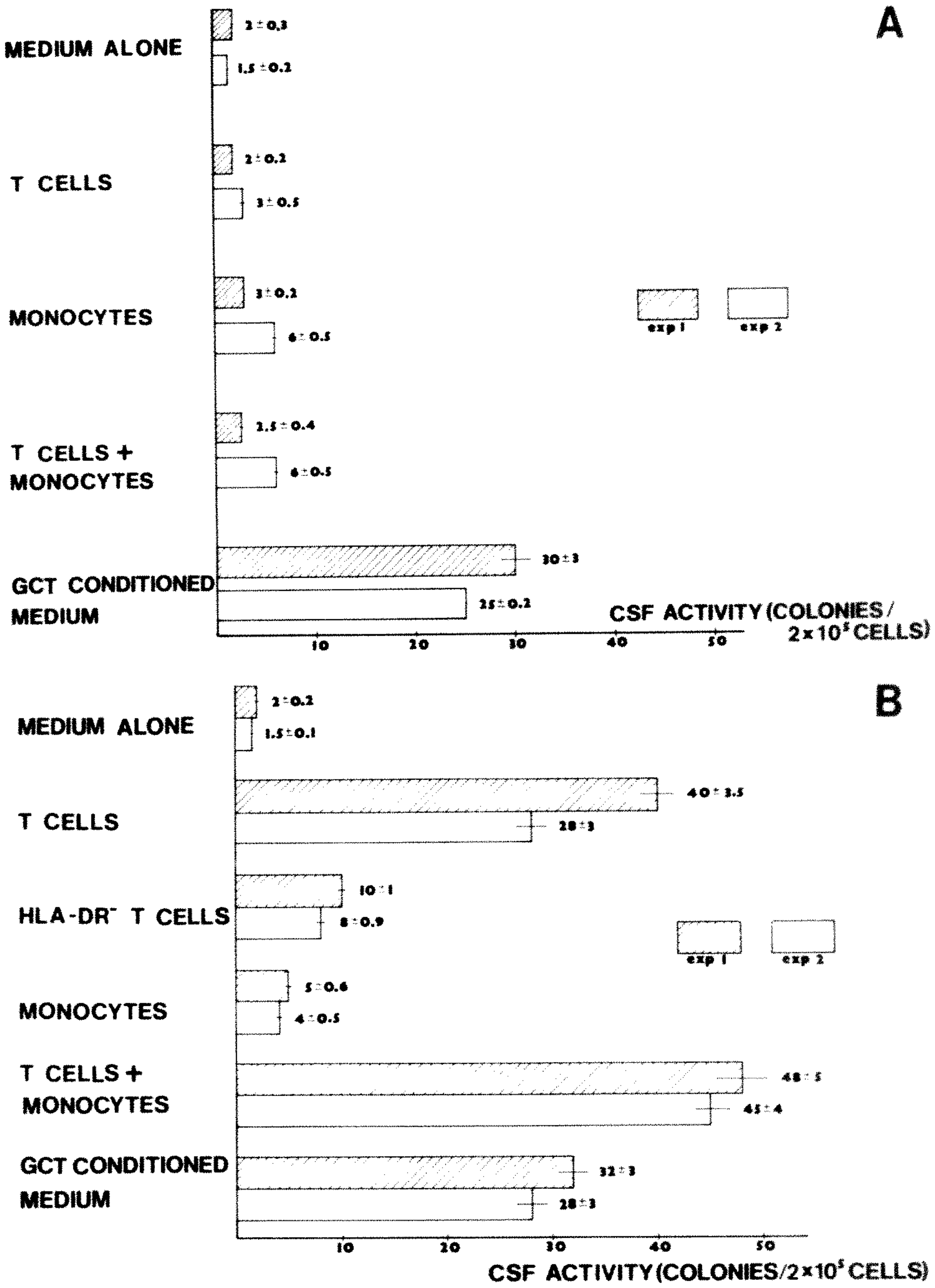

Fig 1. CSA production by T cells and monocytes, cultured alone or in combination. T cells and monocytes, recovered following adherence (90 ± 5 OKM1$^+$ cells), were cultured for 48 hours in the absence (A) or in the presence (B) of PHA, alone (1×10^6 cells/mL) or in combination (1×10^6 T cells with 3×10^5 monocytes). Culture supernatants were tested for CSF activity on peripheral blood MNC depleted of both E rosetting cells and adherent cells. Results are mean ± SE of triplicate cultures/2×10^5 cells seeded.

exogenous stimuli. Figure 3 shows that CSA was released predominantly by low density, activated B cells. In order to confirm a correlation between CSA release and B-cell activation, lymphocytes from the fourth Percoll fraction (small B cells) were cultured with and without SAC for 48 hours. CSA was detected only in culture supernatants of SAC-stimulated B cells (Fig 4).

B cells from B-CLL patients release CSA in vitro. B cells from twelve B-CLL patients were tested for CSA production. B cells were isolated from peripheral blood MNC by removal of E rosetting cells followed by treatment with the OKM1 monoclonal antibody and complement. This method yielded cell preparations containing almost exclusively B cells (Table 2).

B cells from nine cases consistently produced CSA in unstimulated cultures when tested on different occasions, whereas cells from the remaining cases proved consistently negative for CSA production.

Figure 5 shows one determination per case tested and demonstrates variations in the amount of CSA produced by the various cases.

CSA from B-CLL cells induced the growth of two types of colonies, ie, colonies that comprised monocytes-macrophages exclusively and colonies that contained neutrophils and

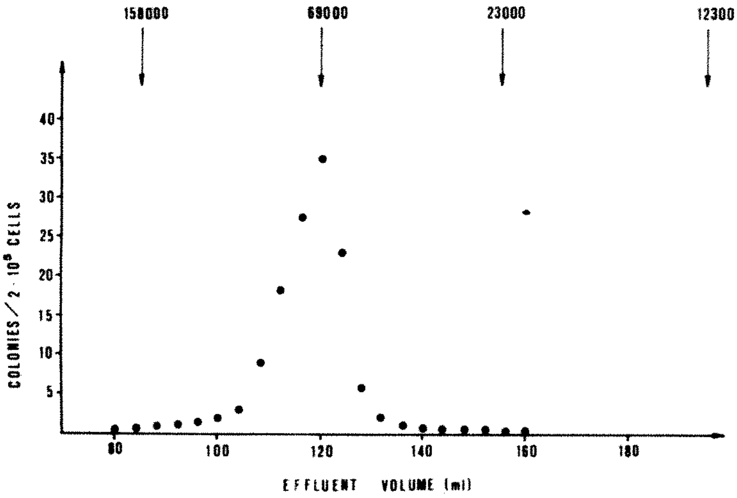

Fig 2. Elution pattern of CSF activity produced by normal B cells. Culture supernatants were ammonium sulphate-precipitated and filtered on a Sephadex G-75 column: fractions were collected and aliquots of each fraction were assayed for CSF activity.

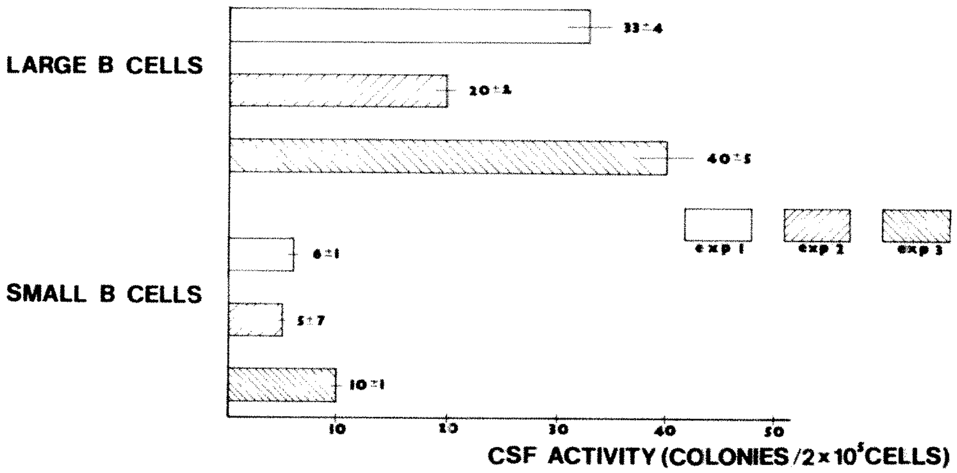

Fig 3. CSA production by small and large tonsil B cells. Purified tonsil B cells were fractionated on a Percoll density gradient. Large and small B cells were cultured for 48 hours in RPMI-FCS; their supernatants were tested using peripheral blood target cells. Results are mean ± SE of triplicate cultures/2 × 10^5 cells seeded.

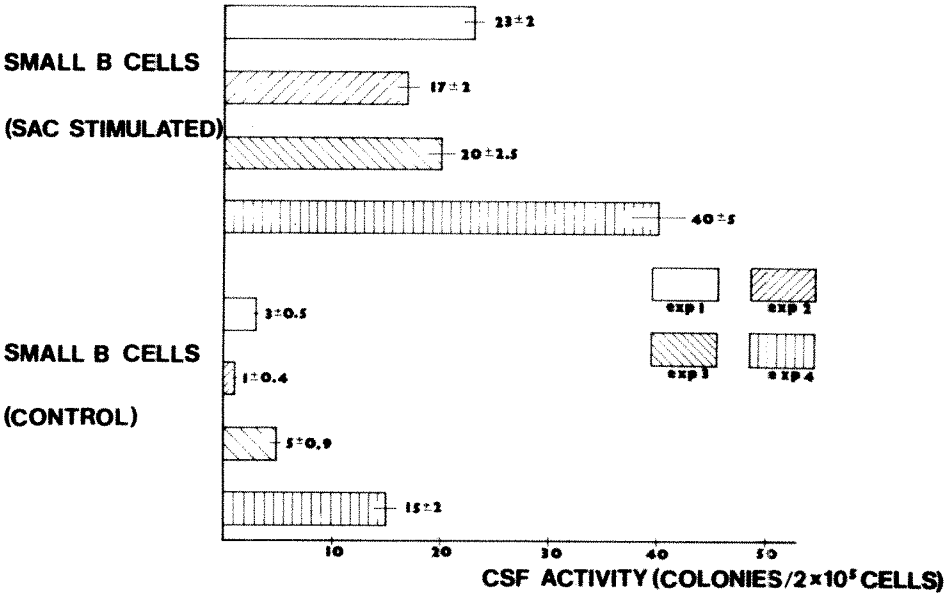

Fig 4. CSA production by small tonsil B cells stimulated with SAC. Small, resting B cells were cultured for 48 hours in the presence or in the absence of SAC. Culture supernatants were subsequently tested on peripheral blood target cells. Results are mean ± SE of triplicate cultures/2 × 10^6 cells seeded.

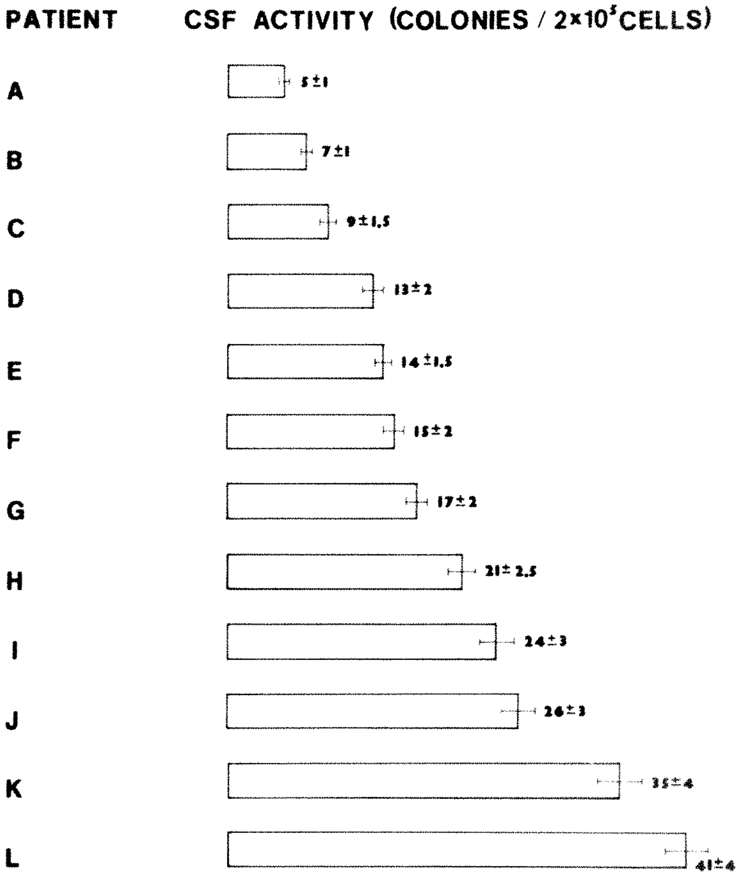

Fig 5. CSA production by B-CLL cells. Purified B cells were cultured for 48 hours and their supernatants tested on peripheral blood target cells. Results are mean ± SE of triplicate cultures/2 × 10^5 cells seeded. Supernatants from different B-CLL cell suspensions were tested on the same target. The control values, ie, without addition of any supernatant, were 4 ± 1 colonies/2 × 10^5 cells. Therefore, any value below 10 colonies/2 × 10^5 cells was considered to indicate the absence of CSA production.

macrophages. Colonies of the former type were, however, predominant in 14-day cultures of peripheral blood precursors.

DISCUSSION

This study demonstrates that highly purified normal B cells release CSA in culture without apparent stimulation. When B cells were fractionated on Percoll density gradients, only large, low-density cells that expressed activation markers were found to release CSA, thus suggesting that CSA production was a property of in vivo preactivated B cells. Since activation of B cells in vivo requires exposure to antigen, an intriguing implication of these results would be that CSA release is under the control of specific (antigen-driven) recognition mechanisms. In this respect the mode of CSA production by B cells would not differ from that of T cells, which also need to be stimulated. T cells require the additional help of accessory cells,[26-28] which present the antigen[29] and supply IL-1.[30] Consistent with the notion that activated B cells produce CSA is the finding that stimulation of resting B cells with SAC induced CSA production. Upon infection with Epstein-Barr virus (EBV) normal B cells release different hemopoietic factors, including CSA[31]. There is ample evidence that EBV-infected cells are in a state of activation[32]; therefore, our findings agree with these data.

The low number of monocytes and T cells that contaminated the B-cell suspensions makes it unlikely that they were responsible for CSA production in our experimental system. This hypothesis is also ruled out by additional control experiments. Thus, when monocytes and T cells were tested, CSA was detected only in the supernatants of cultures that contained mixtures of T cells and monocytes. Furthermore, these cells failed to release CSA spontaneously and had to be stimulated with PHA.

Purified B cells from nine out of 12 B-CLL patients consistently produced CSA in vitro in the absence of any stimulus. Recent studies have demonstrated that, in the individual B-CLL clones, there are a number of maturing,[33-36] possibly activated[37] cells, the proportion of which varies from case to case. One explanation for the differences in CSA production by cells from the various patients may be related to the different content in the number of activated B cells within each malignant clone. In the individual cases, however, no relationship could be established between CSA release and other features, such as lymphocyte count, treatment, and stage of disease.

The available data do not permit assessment of the type of CSF produced by B cells. Although the apparent mw of B-cell derived CSA (65 to 70 kd) is similar to that reported for CSF-1,[8] other features argue against this possibility. For example, unlike CSF-1, CSA released by B cells supports the growth of both macrophage and mixed colonies and is much more effective on human than on mouse bone marrow cells. Furthermore, the biochemical characterization should be considered as preliminary; therefore, comparisons are not possible on these grounds.

B-cell supernatants were tested for CSA mainly on peripheral blood target cells largely depleted of T cells and monocytes. The experimental system used suggests two interpretations for the data, namely, (a) B cells could release a factor acting directly on myeloid precursors, ie, a bona fide CSF, or (b) B cells could release a factor that triggers other accessory cells present in the suspensions to produce CSF. For example, some of the remaining monocytes or the large granular lymphocytes could be good candidates for this accessory function. In this connection it is worth mentioning that monocyte-derived soluble factors stimulate CSA release by endothelial cells.[14]

Besides promoting proliferation and maturation of myeloid progenitors, CSFs also exert a regulatory activity on some effector functions of fully differentiated myeloid cells. CSFs potentiate the cytotoxic capacity of macrophages[2,3] and of granulocytes,[4,7] induce macrophage proliferation,[5] and promote release of prostaglandins,[38] plasminogen activator,[39] and of IL-1.[6] By demonstrating the release of CSA by B cells, our results suggest that B cells may play a role in promoting hematopoiesis and in the initiation of the inflammatory process. The latter hypothesis is also in line with the previous findings that both normal[18,40] and malignant[18] B cells release IL-1, a potent mediator of the inflammatory and of the acute phase responses. IL-1 and CSF activities found in B-cell conditioned media are apparently mediated by different molecules, separated on the basis of their mw (IL-1 mw = 20 to 22 kd[18,40]; CSF mw = 65 to 70 kd). Interestingly, however, B-CLL cells, when capable of releasing CSA, also produce IL-1 (Pistoia et al, unpublished results), suggesting, perhaps, that the two factors are often needed synergistically.

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Immunoradiometric Quantitation of Tissue Plasminogen Activator–Related Antigen in Human Plasma: Crypticity Phenomenon and Relationship to Plasma Fibrinolysis

By Tze-Chein Wun and Anne Capuano

A two-site immunoradiometric assay for tissue plasminogen activator (tPA) antigen has been developed using immunoaffinity purified antibody. Various treatments enhanced the detection of tPA antigen in the plasma samples. Maximum detection was obtained by acidification of plasma to pH 4.8 to 6.5 or addition of 0.5 mol/L of L-lysine or L-arginine. Acidification or addition of lysine to plasma is also required for maximum immunoadsorption of plasma tPA antigen on anti-tPA-Ig-sepharose. These results indicate that plasma tPA antigen is partially cryptic to antibody in untreated plasma. The plasma tPA antigen isolated by immunoadsorption of either untreated plasma or acidified plasma on anti-tPA-Ig-sepharose consists mainly of a 100-kd plasminogen activator species as determined by fibrin-agar zymography. The 100-kd activity is possibly a tPA:inhibitor complex. A standardized sample preparation method was conveniently adopted by mixing 3 vol of plasma and 1 vol of 2 mol/L of L-lysine for the assay. Reconstitution and recovery studies showed that the method is specific and permits full detection of both free tPA and tPA:inhibitor complex. The validity of the assay is further supported by the finding that the spontaneous plasma fibrinolysis previously demonstrated to be dependent on plasma tPA antigen is correlated with tPA antigen content. Using the standardized assay, we found that tPA antigen concentrations in 16 blood bank plasmas are equivalent to 3.7 to 20 ng of 60 kd tPA/mL. In all the plasma tested, more than half of the antigen is undetected unless the plasma is treated as described above.

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HUMAN PLASMA contains a plasmin-dependent fibrinolytic system. Initiation of the system involves plasminogen activator-catalyzed conversion of plasminogen into plasmin, which in turn degrades fibrin into soluble degradation products (for review, see references 1 and 2).

Although several plasma proteins, including tissue type plasminogen activator (tPA),[3] urokinase (uPA),[4,5] and factor XII/kallikrein[6] have been shown to be capable of activating plasminogen, more recent results indicate that tPA may be the primary initiator of fibrinolysis in the plasma milieu. These results include: (a) Spontaneous fibrinolysis in human plasma in vitro is solely dependent on tPA but not on uPA or factor XII/kallikrein[7]; (b) plasma fibrinolysis is highly correlated with the tPA concentration within the known physiological range of tPA[7]; (c) tPA possesses high affinity for fibrin, and its activity is greatly potentiated by fibrin;[8,9] and tPA is capable of eliciting plasma fibrinolysis without systemic activation of plasminogen in the serum.[10]

The tPA antigen concentration in plasma is very low, in the nanogram/milliliter range, and most of the antigen appears to be complexed to a plasma inhibitor.[7] For these reasons, immunoassays appear to be more suitable for measuring total tPA content of plasma. In developing an immunoradiometric assay for tPA, we observed that plasma tPA appeared to be partially cryptic and subsequently discovered simple ways to overcome this problem. An assay method has been devised to allow full detection of plasma tPA antigen. Furthermore, we found that the tPA content determined by this assay correlates well with the spontaneous fibrinolytic activity of a series of randomly selected plasma samples, suggesting that quantitation of plasma tPA is a useful measure of in vitro fibrinolytic potential.

MATERIALS AND METHODS

tPA and tPA antiserum. Purified tPA was isolated from the media of HeLa cells conditioned in 10 ng/mL of 12-*O*-tetradecanoyl-phorbol-13-acetate, 0.2% lactalbumin hydrolysate, and 50 kallikrein inhibitor U/mL of aprotinin in Dulbecco's modified Eagle's medium (DMEM) by one-step affinity purification on an *Erythrina latissima* inhibitor-Sepharose 4B column.[11] The purified HeLa tPA is indistinguishable from tPA isolated from melanoma cell cultures by biochemical and immunological criteria[7] and consists mainly of single-chain form. Antiserum against tPA was obtained by immunizing rabbits with 100 μg of purified HeLa tPA in Freund's complete adjuvant and boosting them each month thereafter with 50 μg of the same protein in incomplete adjuvant.

Miscellaneous. The 96-well polyvinyl microtiter plate was obtained from Dynatech, Alexandria, VA; bovine γ globulin and serum albumin were obtained from Miles Laboratories, IN; chloramine T, EDTA, benzamidine, L-arginine, L-lysine, 6-aminohexanoic acid (EACA), poly-L-arginine, poly-L-lysine, Tween 80, and human thrombin (3,000 NIH U/mg) were from Sigma, St Louis; ^{125}I-sodium iodide was from Amersham, Arlington Heights, IL; Sephadex G25 and CNBr-activated sepharose 4B were from Pharmacia, Piscataway, NJ; human fibrinogen (Kabi, grade L) was from Helena Laboratories, Beaumont, TX; fresh blood bank plasma was obtained from New York Blood Center; porcine heparin (162 U/mg) was from Calbiochem, La Jolla, CA.

Plasma. Fresh, platelet-rich plasma (PRP), obtained from the New York Blood Center, was centrifuged at 3,300 g for 10 minutes at 4°C to separate the platelets. The supernatant was further centrifuged at 16,300 g for 1 hour at 4°C to obtain platelet-free plasma (PFP). The PFP was then aliquoted and stored at −65°C. Individual blood samples were obtained from apparently healthy volunteers with or without venous occlusion. Prevenous occlusion blood samples were obtained by venipuncture with minimum handcuff after the donors rested in a sitting position for at least 5 minutes. Postvenous occlusion bloods were obtained by inflating a sphyg-

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momanometer around the right arm to halfway between systolic and diastolic pressure for 5 minutes, and taking the blood before deflating the cuff. Nine-milliliter samples of blood were collected into 1 mL of 3.8% sodium citrate on ice. The blood was centrifuged at 350 g for 10 minutes to obtain PRP. The latter was further centrifuged in an Eppendorf microfuge for 5 minutes at 4°C to obtain PFP and stored at -65°C. In this study, pooled plasma was prepared by mixing equal volumes of individual plasma from four to eight persons.

Purification of antibody against tPA. Specific antibody against tPA was isolated from antiserum by immunoaffinity chromatography on a tPA-Sepharose 4B column as described previously.[7]

Iodination of anti-tPA-Ig. Specific antibody against tPA was iodinated by a gentle chloramine T method. Fifty microliters of anti-tPA-Ig (1.74 mg/mL in 0.15 mol/L of NaCl) was mixed with 25 μL 0.5 mol/L of $NaPO_4$ pH 7.4, 35 μL of 0.093 mg/mL of chloramine T ($\sim$20-fold molar excess over antibody), and 5 μL of 100 mCi/mL of ^{125}I-sodium iodide. The mixture was kept on ice for 30 minutes and then chromatographed on a 3-mL Sephadex G-25 (fine) column equilibrated in phosphate-buffered saline (PBS)–0.02% NaN_3. The method gives specific radioactivity of $\sim 3.5 \times 10^6$ cmp/μg of protein.

Sandwich immunoradiometric assay for tPA antigen. Sandwich immunoradiometric assay (IRMA) was carried out in 96-well flexible plastic plates. A 2 μg/mL solution of anti-tPA-Ig was freshly prepared in PBS. Two hundred microliters of the solution was distributed into the wells and allowed to stand at room temperature for 3 hours. The solutions in the wells were removed by suction and were replaced with 300 μL of PBB solution [PBS containing 5 mg/mL of bovine serum albumin (BSA) and 2.5 mg/mL of bovine γ globulin]. These were allowed to stand at room temperature for at least 3 hours. The wells were then washed three times with PBS containing 0.1% Tween 80 and three times with PBS. For IRMA, undiluted citrated plasma or plasma diluted 3:1 either with water or with 2 mol/L of lysine were pipetted into the wells. Reference standard solutions containing 0, 1, 2, 4, 8, and 16 ng/mL of tPA were prepared in PBB. The samples and standards (200 μL) were incubated in the wells at 4°C for 24 hours. The wells were then washed three times with a solution containing 0.1 mol/L of boric acid, 25 mmol/L of solium-borate, 1 mol/L of NaCl and 0.1% Tween-80 pH 8.3; three times with PBS-0.1% Tween-80 and three times with PBS. A ^{125}I-anti-tPA-Ig solution (diluted into PBB), containing 100,000 cpm/0.2 mL were added to the wells and incubated at 4°C overnight. The wells were then washed as before and cut out. The radioactivity bound to the wells was counted in a Packard scintillation counter. The IRMA values of duplicated samples were obtained by comparison with the reference standards. Each value represents the average and SE of the measurements.

Quantitation of purified tPA. Purified HeLa cell tPA was quantitated by amino acid analysis based on the amino acid composition deduced from the DNA sequence for tPA[12] as described elsewhere.[7]

Isolation of plasma tPA antigen. Freshly frozen plasma (100 mL) was thawed in a 37°C water bath and incubated at room temperature for >3 hours to ensure formation of tPA-inhibitor complex. The plasma was titrated to pH 6 with 1 N HCl and passed through a 3-mL anti-tPA-Ig Sepharose 4B column (prewashed with 0.15 mol/L of NaCl). The column was then washed with 30 mL of PBS supplemented with 0.4 mol/L of NaCl, 0.1 mol/L of benzamidine, and 1% Triton X-100 and then with 30 mL of the same buffer without Triton X-100 (Sigma, St Louis). The material bound to the column was eluted with 0.1 mol/L of glycine/HCl pH 2.2, concentrated, and dialyzed against the same buffer using a collodion bag. The eluate contains essentially only 100-kd plasminogen activator activity as analyzed by fibrin-agar zymography.[7]

RESULTS

Two-site IRMA of tPA in buffer and in plasma: Effect of additives. When purified HeLa tPA was dissolved in PBB solution and used as a standard for IRMA as described in the experimental section, a linear relationship between the concentration of tPA (0 to 16 ng/mL) and the radioactivity bound to the microtiter wells was observed (Fig 1). Nonlinearity occurred when higher concentrations of tPA were used. This method has a sensitivity of $\sim$0.5 ng/mL of tPA antigen. When the same IRMA protocol was carried out using a citrated plasma diluted 3 to 1 with PBB and applied to the wells, the plasma tPA content was equivalent to 2.7 ± 0.25 ng/mL (arrows, Fig 1). We also assayed the same citrated plasma supplemented with 5 mmol/L of EDTA. The radioactivity bound to the well showed that it was equivalent to 6.5 ± 0.28 ng/mL. In contrast, the reference standard remained essentially the same when supplemented with 5 mmol/L of EDTA. This increase in detection of tPA-related antigen in the EDTA-plasma over that in citrated plasma suggested that plasma tPA-related antigen may be masked by some plasma component, and compounds such as EDTA can to some extent unmask the tPA. Based on this assumption, we tested various compounds, most of which interact with the fibrinolytic and coagulation systems. Table 1 summarizes the comparison of the effects of L-lysine, EDTA, EACA, benzamidine, Tween-80, sodium acetate buffer, pH 5.2, L-arginine, poly-L-lysine, poly-L-arginine, and heparin on the IRMA of tPA in four different pooled plasma samples. In all plasmas, 0.5 mol/L of L-lysine invariably gave the highest IRMA values and acidifications of plasma to pH 5.2 or addition of 0.5 mol/L of L-arginine yielded the same result.

Effect of L-lysine and acidification on the binding of plasma tPA antigen to anti-tPA-Ig. To characterize the masking phenomenon in more detail, we selected L-lysine and acidification for further study. Figure 2 shows the effect

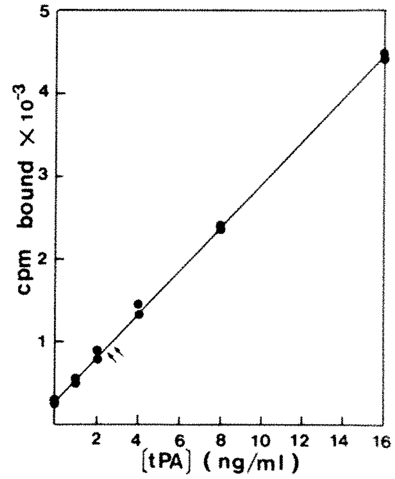

Fig 1. Immunoradiometric assay (IRMA) of tissue plasminogen activator (tPA). IRMA was carried out as described in the Materials and Methods section. Straight line, radioactivity bound to the assay wells in relation to the concentrations of tPA (in duplicate). Arrows, assay value of tPA antigen in an untreated pooled plasma sample.

Table 1. Effect of Various Additives on the IRMA of tPA Antigen in Pooled Plasma

Samples*	tPA Antigen (ng/mL)†
Plasma A + H_2O	2.7 ± 0.10
EDTA (5 mmol/L)	6.5 ± 0.28
EDTA (63 mmol/L)	7.5 ± 0.57
EACA (0.5 mmol/L)	6.7 ± 0.10
L-lysine (0.5 mmol/L)	12.3 ± 1.5
Plasma B + H_2O	4.0 ± 0.15
Tween 80 (0.1%)	4.4 ± 0.35
Benzamidine (0.1 mol/L)	8.0 ± 0.40
Na acetate, pH 5.2 (0.1 mol/L)	9.0 ± 0.51
L-lysine (0.5 mol/L)	9.0 ± 0.43
Plasma C + H_2O	5.9 ± 0.85
L-arginine (0.5 mol/L)	14.5 ± 0.28
Poly L-lysine (5 mg/mL)	12.8 ± 0.28
Poly L-arginine (5 mg/mL)	8.9 ± 0.35
L-lysine (0.5 mol/L)	14.8 ± 0.21
Plasma D + H_2O	7.2 ± 0.61
Heparin (2.5 U/mL)	7.7 ± 0.42
Heparin (5 U/mL)	4.9 ± 0.28
Heparin (10 U/mL)	14.3 ± 1.2
Heparin (20 U/mL)	12.0 ± 1.1
Heparin (50 U/mL)	7.3 ± 0.52
L-lysine (0.5 mol/L)	20.0 ± 0.50

IRMA, immunoradiometric assay; tPA, tissue plasminogen activator; PBB, phosphate-buffered saline containing bovine γ globulin (2.5 mg/mL) and bovine serum albumin (5 mg/mL).

*Samples were prepared by mixing 3 vol of citrated plasma with 1 vol of H_2O or solutions of various compounds. The final concentrations of the compounds are as indicated.

†IRMA values of tPA antigen concentrations were obtained by comparison to tPA standards dissolved in PBB. All values have been corrected for dilution.

of various concentrations of L-lysine on the amount of plasma tPA antigen detected in the IRMA in comparison with the standard tPA dissolved in PBB. In this particular plasma, the amount of plasma tPA detected in the presence of 0.5 mol/L of L-lysine was 250% of that detected in the absence of L-lysine. In contrast, L-lysine has almost no effect on the IRMA value for standard tPA dissolved in PBB. Thus, more than half of tPA antigen was undetectable by IRMA of the plasma samples. The effect of pH on the IRMA of tPA in plasma is shown in Fig 3. Maximum values were obtained throughout pH 4.8 to 6.7. Above pH 6.7 there was a sharp decline in the value. Because the IRMA value is related to the binding of tPA antigen to the antibody-coated surface, we also tested the effects of acidification and L-lysine on the binding of plasma tPA to our anti-tPA-Ig-Sepharose 4B column. Table 2 summarizes the result of passage of plasma over an anti-tPA-Ig-Sepharose 4B column under various conditions. One time passage of an untreated plasma over anti-tPA-Ig-Sepharose 4B results in the reduction of plasma tPA content to 57%. A second passage of the plasma through the same column results in only a small decrease in tPA content to 44%. By acidifying the flow-through plasma and passing it through the column again, essentially all the tPA-related antigen is depleted; only 2% remains. When a separate, untreated plasma was made 0.5 mol/L of L-lysine and passed through the anti-tPA-Ig-Sepharose once, the tPA content was reduced to 7% of the original plasma sample. These results indicate that part of the plasma tPA antigen is not accessible for binding by the antibody against tPA and that crypticity of plasma tPA antigen with regard to the antibody is possibly due to the masking of tPA antigen by other plasma components.

Identification of plasma tPA-related antigens. The above experiments suggest that two pools of plasma tPA-related antigen exist, one accessible to anti-tPA-Ig and one not. To discover whether these two pools of tPA antigen represent different molecular species, we made a zymo-

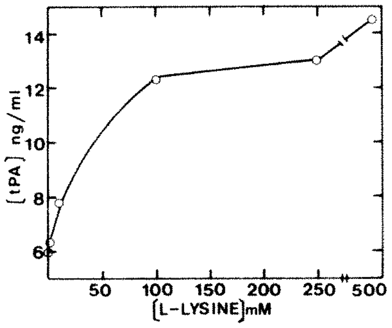

Fig 2. Effect of L-lysine on the immunoradiometric assay (IRMA) of tissue plasminogen activator (tPA). A pooled plasma was diluted 3 to 1 with H_2O or L-lysine such that the final concentration of lysine in the mixture was as indicated in the graph. The concentrations of tPA antigen were obtained by comparison with standard tPA dissolved in phosphate-buffered saline containing bovine γ globulin (2.5 mg/mL) and bovine serum albumin (5 mg/mL).

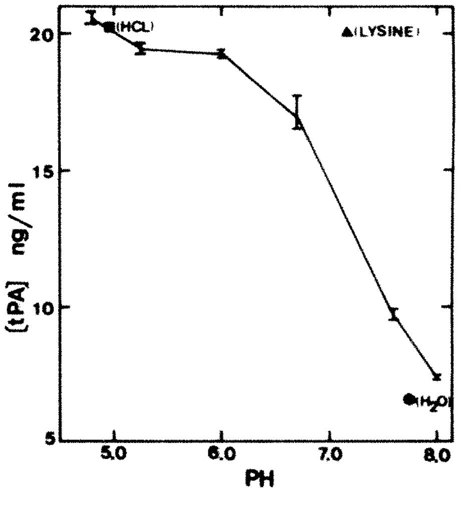

Fig 3. Effect of plasma pH on immunoradiometric assay (IRMA) of tissue plasminogen activator (tPA). Pooled citrated plasma was diluted with 1/8 vol of 1 mol/L of sodium acetate, pH 3.5 to 8.0 such that the final pH of the mixtures ranged from 4.8 to 8.0. The concentrations of tPA antigen were obtained by comparison with standard tPA dissolved in phosphate-buffered saline containing γ globulin (2.5 mg/mL) and bovine serum albumin (5 mg/mL) (PBB) (in duplicate). tPA antigen concentration in plasma diluted with H_2O in the same volume ratio (●) (final pH 7.75). Value of tPA antigen concentration in plasma diluted with L-lysine (final concentration 0.5 mol/L, pH 7.2) (▲); value of tPA antigen concentration in plasma diluted with ⅛ vol of 0.4 mol/L of HCl (final pH 4.95) (■).

Table 2. Effect of Acidification and Addition of L-Lysine on the Binding of Plasma tPA to Anti-tPA-Ig-Sepharose 4B

Samples*	tPA Antigen (ng/mL)	tPA Antigen % tPA
1. Plasma, before passing anti-tPA-Ig-Sepharose	12.4 ± 0.07	100
2. Flow-through from passage of plasma (1) through anti-tPA-Ig-Sepharose	7.0 ± 0.14	57
3. Flow-through from passage of flow-through (2) through anti-tPA-Ig-Sepharose a second time	5.2 ± 0.28	44
4. Flow-through (3) titrated to pH 6, then passed through anti-tPA-Ig-Sepharose	0.27 ± 0.02	2
5. Flow-through from passage of plasma adjusted to 0.5 mol/L of L-lysine through anti-tPA-Ig-Sepharose	0.87 ± 0.09	7

Abbreviations as in Table 1.

*Chromatography was carried out on a 0.6 × 3 cm anti-tPA-Ig Sepharose 4B column at room temperature. The flow rate was ~0.4 mL/min in all runs.

graphic comparison. The eluate obtained from anti-tPA-IG-Sepharose chromotography of untreated plasma represented tPA antigen that was freely accessible to antibody. The effluent, containing the cryptic tPA-related antigen, was acidified and rechromatographed. The tPA-related antigen was completely bound this time and, upon elution, constituted the cryptic tPA antigen. Figure 4 shows that no detectable difference existed between the two. Both contained mainly a plasminogen activator with a mol wt of 100 kd. As we demonstrated previously,[7] the 100-kd species appears to be a complex formed by the 60-kd tPA with a plasma inhibitor.

A standardized IRMA for quantitating plasma tPA-related antigen. Although addition of 0.5 mol/L of L-lysine appears to give the highest value of tPA antigen by IRMA, a recovery study was required to ascertain that this treatment of plasma does indeed reveal the full amount of tPA. To examine recovery, we carried out experiments to compare IRMA values for purified 60-kd tPA and partially purified plasma tPA antigen (obtained as described in the Materials and Methods section) reconstituted into PBB buffer or tPA-depleted plasma. Table 3 summarizes the results. When the same amount of partially purified plasma tPA antigen was reconstituted into the same volume of PBB or plasma, the IRMA values of plasma were only about one-half that in PBB, indicating that part of the added plasma tPA antigen was masked by plasma. Addition of 0.5 mol/L of L-lysine to the reconstituted plasma gave essentially the same value as that in PBB buffer. When the same amount of HeLa 60-kd tPA was reconstituted into the same volume of PBB or plasma, the IRMA value in plasma was also less than that in PBB, although masking appeared not to be as extensive as that for the plasma tPA antigen. Addition of 0.5 mol/L of L-lysine to the plasma again allowed detection of the full amount of tPA antigen. These results indicate that 0.5 mol/L L-lysine fully unmasked both tPA and plasma tPA:inhibitor complex. We therefore adopted addition of 1 vol of 2 mol/L of L-lysine to 3 vol of plasma as a convenient step in a standard IRMA for tPA antigen. Using this protocol, we assayed 16 individual or pooled plasmas obtained from a blood bank. As shown in Table 4, the plasma tPA antigen varied quite widely among individuals (3.7 to 20 ng/mL).

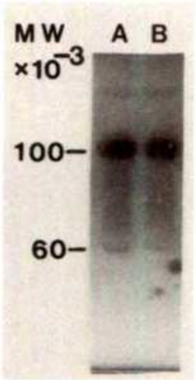

Fig 4. Zymography of plasma tissue plasminogen activator (tPA) antigen bound to anti-tPA-Ig-Sepharose 4B under neutral and acidic conditions. A freshly frozen citrated pooled plasma was thawed, incubated at room temperature for 5 hours and passed twice through an anti-tPA-Ig-Sepharose 4B column (3 mL) pre-equilibrated in 0.15 mol/L NaCl, 0.02% NaN_3. The column was washed with 30 mL of phosphate-buffered saline (PBS), 0.4 mol/L of NaCl, 0.1 mol/L of benzamidine, 1% Triton X-100 and 30 mL of the same buffer without Triton X-100. The tPA bound to the column was eluted in 6 mL of 3 mol/L of NaSCN. The eluate was supplemented with 1 mg bovine serum albumin (BSA) and 1 µL of Tween 80; concentrated to 1 mL, and dialyzed against 50 mmol/L of Na-acetate, pH 3.5 (eluate 1). The plasma that flowed through the column was titrated with 1.5 N HCl to pH 6.0 and then passed through the column again. The column was washed and eluted as described above. The eluate was concentrated and dialyzed as before (eluate 2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymography of the eluates were performed as described in the Materials and Methods section; (A) 10 µL of eluate 1; (B) 15 µL of eluate 2.

Correlation of tPA antigen content and spontaneous fibrinolysis in plasma in vitro. As we showed previously,[7] addition of anti-tPA-Ig to human plasma causes essentially complete inhibition of spontaneous fibrinolysis, indicating that plasma tPA-related protein is required for initiation of fibrinolysis in the plasma in vitro and that in plasma fibrinolytic activity is directly related to its tPA content. Therefore, we studied by IRMA the rates of spontaneous fibrinolysis in

Table 3. IRMA for tPA Antigen in PBB Buffer and tPA-Depleted Plasma

tPA Sample	IRMA Value PBB	IRMA Value Plasma/H_2O*	IRMA Value Plasma/L-Lysine†
Plasma tPA antigen	21.5	11.4 ± 0.85	21.3 ± 0.07
	10.9	5.5 ± 0.41	10.9 ± 0
HeLa 60-kd tPA	24.1	20.0 ± 1.08	24.7 ± 0.32
	12.1	9.6 ± 0.45	12.8 ± 0.57

Abbreviations as in Table 1.

*tPA antigen dissolved in a mixture of 3 vol of plasma and 1 vol of H_2O.

†tPA antigen dissolved in a mixture of 3 vol of plasma and 1 vol of 2 mol/L of L-lysine.

Table 4. IRMA for tPA Antigen in Various Plasma

Plasma/H_2O (ng/mL)*	Plasma/L-lysine (ng/mL)†	Plasma/L-Lysine / Plasma/H_2O
4.7	9.3	1.98
2.0	4.8	2.40
7.6	16.2	2.13
5.2	12.0	2.31
1.9	4.0	2.11
2.8	7.9	2.82
3.7	8.6	2.32
3.2	6.8	2.13
5.2	9.4	1.81
1.5	4.0	2.67
2.2	3.7	1.68
4.9	11.1	2.27
3.0	8.6	2.87
2.7‡	12.3	4.56
5.9‡	14.8	2.50
7.2‡	20.0	2.78

Abbreviations as in Table 1.

*IRMA values of tPA antigen measured in mixtures of 3 vol of plasma and 1 vol of H_2O.

†IRMA values of tPA antigen measured in mixtures of 3 vol of plasma and 1 vol of 2 mol/L of L-lysine.

‡Pooled plasma (from four individuals) was used.

several different plasmas that had a diverse amount of tPA antigen. Figure 5A shows the time course of the lysis of several plasmas. When the tPA content of these plasmas was plotted against their rate of lysis, taken as the inverse of the time required for 50% lysis of the clot, $1/S_{50}$, a linear relationship with a correlation coefficient of $r = .972$ was found (Fig 5B). This result further substantiates the significance of tPA as the determining factor in initiating fibrinolysis and indicates that the measurement of tPA faithfully reflects the fibrinolytic potential of different plasmas in vitro.

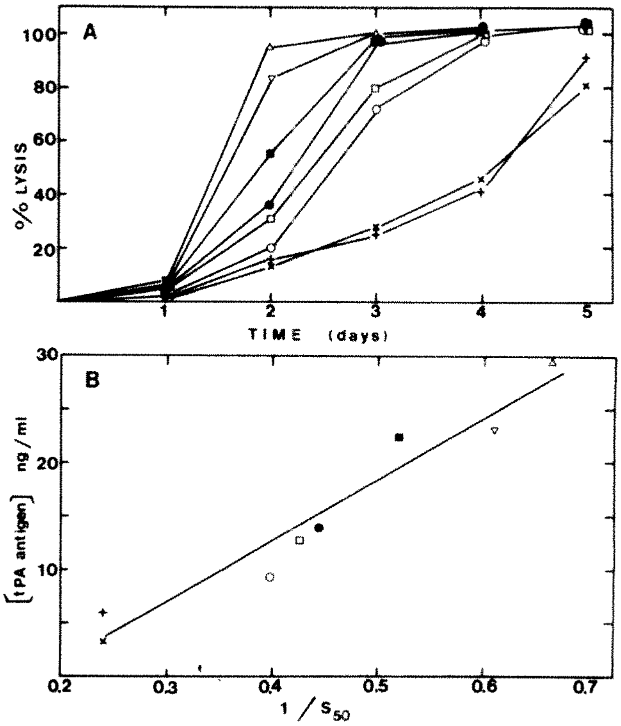

Fig 5. The relationship of the rate of clot lysis to the tissue plasminogen activator (tPA) antigen content in various plasma samples. (A) Time course of clot lysis in various plasma samples. (B) Rates of clot lysis ($1/S_{50}$) v tPA antigen concentration in various plasma samples; (Δ and ∇) plasmas from two patients with liver cirrhosis; (■) T. W., postocclusion plasma; (□) T. W., preocclusion plasma; (●) S.P., postocclusion plasma; (○) S.P., preocclusion plasma; (×) J. A., preocclusion plasma; (+) A. C., preocclusion plasma. The tPA antigen concentration in the plasma samples was measured by immunoradiometric assay in the presence of 0.5 L-lysine. The correlation coefficient, γ, was calculated as 0.972.

DISCUSSION

Several tPA immunoassay systems have been described.[13-17] A major common problem in these assays is that recovery is poor and the dilution curves of tPA in plasma are not parallel with dilution curves in buffer.[18] Our data on the immunoradiometric assay of plasma tPA appear to corroborate these findings and suggest that the interference phenomenon is a property unique to tPA in plasma. First, tPA existing in serum-free cell culture media does not require manipulation for full detection of antigen. Second, addition of L-lysine or acidification does not alter the detectability of other antigens such as placental PA inhibitor in pregnancy plasma (Wun, unpublished observations). Third, the interference is not due to the presence of rheumatoid factors as reported for other antigen assays[19,20] since crypticity of plasma tPA is observed in all plasma samples tested. Fourth, the unmasking effect with L-lysine or acidification also occurs using an enzyme-linked immunoassay (ELISA) system[13] obtained from American Diagnostica, indicating that the phenomenon is not an artifact of our specific assay procedure or reagents.

Although addition of EDTA has been reported to improve the results of the immunoradiometric assay of plasma tPA, our results indicate that it is not as efficient in revealing the plasma tPA antigen as is the addition of 0.5 mol/L of lysine or arginine and slight acidification of plasma; neither are treatments with other chemicals such as EACA, Tween 80, polyarginine, heparin, etc., which do not reveal all the plasma tPA antigen (Table 1).

We compared the sensitivity and specificity of this immunoradiometric assay with a widely used enzyme-linked immunosorbent assay (ELISA) system for tPA[13] that recently became available from American Diagnostica. Greater sensitivity (~0.1 ng/mL) has been confirmed to accompany use of enzyme-labeled antibody as compared with ^{125}I-labeled antibody (~0.5 ng/mL). In using the ELISA system, however, we encountered two problems that required some modification of the procedure. First, tPA-depleted plasma gives a background as high as 50% that of the original plasma, suggesting serious nonspecificity. This problem can be overcome by the use of affinity-purified antibody to coat the assay wells as described in this article instead of using whole Ig fraction. Second, acidification of plasma to pH 3.9 with subsequent reneutralization as recommended by the report[13] gives a tPA value somewhat lower than the addition of 0.5 mol/L of lysine (Wun, unpublished observations), suggesting that acidification and reneutralization is not as effective in revealing plasma tPA antigen as is treatment with lysine.

Our finding of a linear relationship between total plasma tPA antigen and in vitro spontaneous fibrinolysis requires cautious interpretation. In this and a previous article[7] we demonstrated that very little free tPA exists in stored plasma. Plasma samples generally contain excess inhibitor relative to tPA, and the formation of tPA: inhibitor complex in the circulation and/or after blood collection appears to be highly favored. Stored plasma samples nonetheless exhibit low-level spontaneous fibrinolytic activity that is tPA dependent.[7] As shown in the accompanying article,[21] the tPA:inhibitor complex isolated from stored plasma is capable of inducing plasma fibrinolysis that is insensitive to the presence of excess tPA inhibitor, in sharp contrast to free tPA-induced fibrinolysis that is sensitive to inhibition by inhibitor. Therefore, the complex-induced fibrinolysis does not appear to be attributable to residual free tPA. It is probably due to a low-level activity intrinsic to the complex or to slow, localized dissociation of the complex with fibrin added. These explanations are consistent with the finding that, for the bulk of stored plasma samples in which inhibitor is in excess of tPA, the amoun of tPA antigen in the form of tPA:inhibitor complex is related to in vitro spontaneous fibrinolytic activity. Despite in vitro correlation, measurement of total tPA antigen or assay of spontaneous fibrinolytic activity may not be adequate to define the in vivo fibrinolytic state. It is likely that in vivo fibrinolysis may be dynamically determined by the availability of free tPA and its kinetic interaction with inhibitor.[21] These considerations highlight the need for further development of reliable quantitative assays for the inhibitor to define the fibrinolytic state of plasma better.

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Initiation and Regulation of Fibrinolysis in Human Plasma at the Plasminogen Activator Level

By Tze-Chein Wun and Anne Capuano

The initiation and regulation of fibrinolysis has been studied by reconstitution of fibrinolytic activity in human plasma in vitro. Depletion of tissue plasminogen activator (tPA) antigen by immunoadsorption of human plasma with anti-tPA Ig Sepharose 4B leads to total loss of spontaneous fibrinolytic activity determined by lysis of a thrombin-induced clot. Addition of physiological concentrations of purified tPA to tPA-depleted plasma restores fibrinolytic activity as a function of the length of time between tPA addition and clotting. Addition of free tPA to tPA-depleted plasma followed by immediate clotting results in a high rate of fibrinolysis. In contrast, when free tPA is allowed to incubate in plasma for 10 to 60 minutes prior to clot formation, the fibrinolytic activity of tPA is gradually lost. The loss of tPA-induced fibrinolytic activity in unclotted plasma is accompanied by decreased partitioning of tPA antigen into fibrin after clotting and is kinetically correlated with the formation of a 100 kilodalton (kDa) tPA complex as demonstrated by SDS-gel electrophoresis and fibrin-agar zymography. These results suggest that free tPA is susceptible to complexation by the plasma inhibitor in the absence of a clot. Fibrin formation renders tPA relatively inaccessible to inhibition. The tPA antigen isolated from stored plasma consists mainly of 100 kDa activity in SDS-gel electrophoresis and zymography, indicating that the tPA complex is resistent to dissociation by SDS. Upon rezymography of the sliced gel, only a 60 kDa tPA activity is found, suggesting that the activity at 100 kDa is at least partly due to free tPA dissociated from the complex during the first zymography. Conversion of tPA complex to enzymatically active free tPA also occurs with brief SDS exposure followed by incubation in the presence of excess Triton X-100 or by hydroxylamine treatment. These results reconcile the apparent discrepancy of the 100 kDA inhibitor-tPA complex manifesting plasminogen activation activity during zymography. The plasma tPA-inhibitor complex is precipitated strongly by antisera against plasminogen activator inhibitors (PAIs) of human Hep G2 hepatoma and HT-1080 fibrosarcoma cells and weakly by antiserum against bovine aortic endothelial cell PAI but not by antiserum against a placental PAI (PAI-2) suggesting that the plasma inhibitor is immunologically related to Hep G2, HT-1080 and possibly endothedial cell PAIs. Based on the above findings, a simple model for the initiation and regulation of plasma fibrinolysis at the PA level has been formulated.

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FIBRINOLYSIS refers to a process in which fibrin is proteolytically converted into soluble degradation products. The plasminogen activators (PAs) in human plasma appear to play the initiating role in this process by catalyzing the conversion of plasminogen into plasmin, which subsequently degrades fibrin.[1,2]

Because of the exceptionally low[3,4] and labile[5] nature of plasma fibrinolytic activity in vitro, few investigators have directly studied fibrinolysis in plasma. Instead, most efforts have been directed toward study of purified components and euglobulin. The latter approaches, although more facile, have the limitation that important regulatory components are completely or partially eliminated, complicating the resolution of several fundamental questions. In particular, what are the relative roles of the various plasma activators—tissue plasminogen activator (tPA),[6] urokinase (uPA),[7-9] and factor XII/kallikrein[10-12]—in plasma fibrinolysis? Are plasma PA inhibitors truly involved in the regulation of overall plasma fibrinolysis? And, if so, which ones? How is plasma fibrinolysis initiated and regulated?

Our previous work on the human fibrinolytic system has made available several purified PAs, inhibitors, and their specific antibodies.[7,13,14] These together with our recent development of a reproducible plasma fibrinolytic assay[15] have allowed us to investigate the initiation and regulation of plasma fibrinolysis. Using these tools, we have recently demonstrated that in vitro spontaneous plasma fibrinolysis is strictly dependent on tPA-related protein but not on urokinase (uPA) or factor XII/kallikrein[15] suggesting that tPA is possibly the primary initiator of fibrinolysis in the plasma milieu. By sodium dodecyl sulfate (SDS)-gel electrophoresis and fibrin-agar zymography techniques, we further showed that the majority of tPA-related activity in plasma has an apparent molecular weight of 100 kDa, different from free tPA (60 kDa). The 100-kDa activity can be generated by incubating physiological concentration of free tPA in the tPA depleted plasma.[15] These observations suggested to us that plasma contains a component that specifically forms a complex with tPA under normal physiological conditions and the component may function to regulate tPA-dependent fibrinolytic activity.

In this report, we describe a series of reconstitution experiments which demonstrate that formation of the complex is kinetically correlated with decreased partitioning of tPA antigen into fibrin clots and depressed plasma fibrinolytic activity. Furthermore, we have found that regulation of tPA-induced plasma fibrinolysis is dictated by the presence or absence of fibrin. The absence of fibrin renders tPA-dependent fibrinolytic activity susceptible to inhibition, while the presence of fibrin sustains a lasting fibrinolysis

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even though the surrounding serum contains excess inhibitor over added tPA. Based on these and previous results, we propose a simple model that entails key factors involved in the initiation and regulation of plasma fibrinolytic activity at the PA level.

MATERIALS AND METHODS

Materials

Pure, single-chain free tPA (60 kDa) was isolated from HeLa cell culture medium.[15] Antiserum against HeLa tPA was raised in rabbit.[15] Platelet-rich plasma was obtained from New York Blood Center. Thrombin (3,000 NIH units/mg, product #T6759) was from Sigma, St. Louis. Goat antirabbit-Ig beads were obtained from BioRad, Richmond, CA. Other materials used were the same as described in the accompanying paper.[16]

Methods

Plasma. Fresh, acid-citrate-dextrose anticoagulated plasma obtained from the blood bank was centrifuged at 3,300 g for 10 minutes at 4°C to separate platelets. The supernatant was further centrifuged at 16,300 g for 1 hour at 4°C to obtain platelet-free plasma and stored at −65°C.

Anti-tPA-Ig-Sepharose 4B. Specific antibody against tPA was isolated from rabbit antiserum by immunoaffinity chromatography on a tPA-Sepharose column.[15] The specific anti-tPA-Ig was then coupled to cyanogen bromide–Sepharose 4B (5 mg antibody per mL wet gel).[15]

Sandwich immunoradiometric assay of tPA. The immunoradiometric assay was carried out in 96-well flexible plastic plates as described in the accompanying paper.[16] The plasma samples were diluted 3:1 with 2 mol/L L-lysine to ensure full detection of tPA-related antigen.

Plasma clot lysis assay. The clot lysis assay was carried out as described.[15] In brief, plasma was supplemented with ^{125}I-fibrinogen and 0.2% NaN_3 and divided into 95 μL aliquots in microfuge tubes. Five μL of a thrombin solution (20 NIH units/mL in 0.5 mg/mL bovine serum albumin) was then added to clot the plasma in each tube. The clots were incubated at 37°C. At various time intervals, a tube was taken, vortexed, and centrifuged to separate the serum. The percent of clot lysis was calculated based on the amount of ^{125}I-fibrin degradation products released into serum.

SDS-polyacrylamide gel electrophoresis and zymography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed using Laemmli's buffer system.[17] Zymography was carried out by washing the gel with 2.5% Triton X-100 and subsequently laying the gel onto a fibrin-agar layer containing plasminogen.[18]

Preparation of tPA-depleted plasma and isolation of plasma 100-kDa tPA complex. Freshly frozen plasma (100 mL) was thawed in a 37°C water bath and incubated at room temperature for more than three hours to ensure full formation of tPA:inhibitor complex. The plasma is titrated to pH 6 with 1 N HCl and passed through a 3-mL anti-tPA-Ig Sepharose 4B column (prewashed with 0.15 mol/L NaCl). The early flow-through is discarded to avoid dilution. The flow-through pool is essentially depleted of tPA antigen (<0.27 ng/mL by immunoradiometric assay[16]) and is designated tPA-depleted plasma. The anti-tPA-Ig-Sepharose 4B column was washed with 30 mL of phosphate-buffered saline supplemented with 0.4 mol/L NaCl, 0.1 mol/L benzamidine, and 1% Triton X-100 and then with 30 mL of the same buffer without Triton X-100. The material bound to the column was eluted with 0.1 mol/L glycine/HCl, pH 2.2, concentrated and dialyzed against the same buffer using a colodion bag. The eluate contains largely 100-kDa plasminogen activator activity as analyzed by zymography.[15,16]

RESULTS

Plasma tPA Antigen—Existence as 100-kDa Complex Form and Its Formation

Plasma tPA-related antigen can be precipitated from a normal pooled plasma by anti-tPA-Ig. The immunoprecipitated tPA consist of essentially only 100 kDa activity in zymography (Fig 1, lane 5) as opposed to free tPA, which is 60 kDa (lane 6). Plasma tPA antigen can be completely depleted from slightly acidified plasma by anti-tPA-Ig-Sepharose 4B, since no tPA activity is precipitable by anti-tPA-Ig in the immunoadsorbed plasma (Fig 1, lane 1). When free tPA (60 kDa) is added to tPA-depleted plasma, more 100-kDa complex is formed. At near physiological concentrations of tPA (5 to 10 ng/mL), 100-kDa activity is the only prominent complex formed (lanes 2 and 3). At higher tPA concentrations (50 ng/mL), 100-kDa complex is formed together with small amounts of other complexes, but

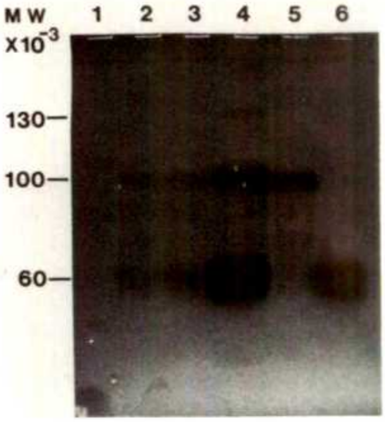

Fig 1. Zymographic study of tPA complex formation in tPA-depleted plasma. Depletion of tPA antigen from plasma was carried out as described in the experimental section. The tPA-depleted plasma (0.18 mL) was mixed with 0.02 mL of 10,000 units/mL aprotinin and 0.01 mL of PBB solution (phosphate buffered saline supplemented with 5 mg/mL bovine serum albumin and 2.5 mg/mL bovine γ globulin) containing 0 to 1,000 ng/mL tPA and incubated for two hours at 37°C to form the tPA complex. To form immunocomplex, 3 μL of 0.2 mg/mL anti-tPA-Ig, 20 μL of 1 mol/L benzamidine and 77 μL of 2 mol/L L-lysine were added and the mixture was incubated at 4°C overnight. Then 25 μL of goat antirabbit Ig bead was added and incubated at room temperature for three hours to precipitate the immunocomplex. The beads were washed four times with a PBS buffer supplemented with 0.4 mol/L NaCl, 0.1 mol/L benzamidine and 1% Triton X-100 and two times with water. Thirty μL of a buffer containing 0.1 mol/L glycine/HCl, pH 2.2, 0.1% SDS, and 6 mol/L urea was added to the washed beads and the mixture was incubated at 37°C for 30 minutes to dissociate the immunocomplex. The mixture was centrifuged briefly in microfuge and 20 μL of supernatant was taken for zymography using 10% polyacrylamide gel. Lane 1, tPA-depleted plasma; lane 2, tPA-depleted plasma incubated with 5 ng/mL tPA; lane 3, tPA-depleted plasma incubated with 10 ng/mL tPA; lane 4, tPA-depleted plasma incubated with 50 ng/mL tPA; lane 5, plasma not depleted of tPA; lane 6, PBB solution incubated with 10 ng/mL tPA.

the majority of tPA remains free (lane 4). These results indicate that normal plasma contains a component that can specifically bind to the free tPA to form a SDS-nondissociable 100-kDa complex, and this plasma component normally exists in limited excess over tPA. Figure 2 shows the zymographic study of the time course of the formation of 100-kDa complex after supplementing a tPA-depleted plasma with 10 ng/mL free tPA. Free tPA activity gradually diminishes and the 100-kDa activity gradually increases with a time course of more than one hour.

Partitioning of tPA Antigens Between Plasma Clot and Serum

When freshly frozen plasma is thawed, incubated at room temperature for several hours, and then clotted by the addition of thrombin, less than 15% of tPA antigen in the plasma is found to be bound to the clot (Table 1). This finding is inconsistent with the known high affinity of tPA for fibrin.[19] To examine the possibility that the low affinity of plasma tPA antigen for fibrin may be due to formation of tPA complex, we have carried out several reconstitution experiments. Introduction of immunoaffinity-isolated tPA complex into tPA-depleted plasma similarly shows low affinity of the complex for the clot. The affinity is not significantly changed by varying the time period prior to clotting (Fig 3B). In contrast, when free tPA is added to tPA-depleted plasma, the affinity of tPA antigen for clot varies

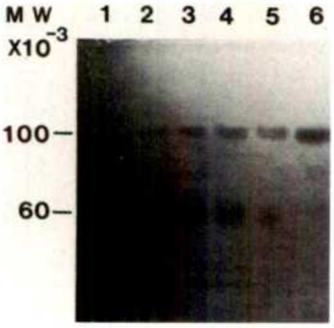

Fig 2. Zymographic study of the time course of the formation of 100-kDa activity following addition of tPA to tPA-depleted plasma. tPA-depleted plasma (1.53 mL) was supplemented with 0.17 mL of 10,000 U/mL aprotinin, cooled on ice for three minutes and mixed with 1.6 μL PBB solution containing 10 μg/mL tPA such that the final concentration of tPA was 10 ng/mL. At 0, 10, 30, 60 and 240 minutes after mixing, 0.3 mL aliquots were taken and added to microfuge tubes containing 0.1 mL of 2 mol/L L-lysine, 0.04 mL of 1 mol/L benzamidine and 0.003 mL of 0.2 mg/mL anti-tPA-Ig. The samples were incubated at 4°C overnight to form immunocomplex. Then, 0.025 mL of goat-antirabbit-Ig beads was added and the suspension was incubated at room temperature for two hours to precipitate the immunocomplex. The beads were washed four times with a PBS buffer supplemented with 0.4 mol/L NaCl, 0.1 mol/L benzamidine and 1% Triton X-100 and two times with water. Thirty μL of a buffer containing 0.1 mol/L glycine/HCl, pH 2.2, 0.1% SDS, and 6 mol/L urea was added to dissociate the immunocomplex. The mixture was centrifuged briefly in a microfuge and 20 μL of supernatant was taken for zymography on a 10% polyacrylamide gel. Lanes 1 to 5, tPA-depleted plasma incubated with tPA for 0, 10, 30, 60 and 240 minutes respectively; lane 6, a normal pooled plasma immunoprecipitated by exactly the same procedure.

Table 1. Immunoradiometric Assay for Endogenous tPA in Plasma and Serum

	tPA Conc. (ng/mL)	% of Plasma tPA
Plasma*	15.8 ± 0.55	100
Serum (citrate)†	13.5 ± 0.10	85
Serum (Ca^{2+})‡	13.9 ± 0.30	88

*A pooled plasma was mixed with $\frac{1}{10}$ volume of aprotinin (10,000 units/mL). Fifty μL of 0.5 mg/mL BSA was added to 0.95 mL of the plasma-aprotinin mixture and used for immunoradiometric assay.

†Serum was prepared by mixing 0.95 mL of the above plasma-aprotinin mixture with 50 μL of 0.5 mg/mL BSA plus 20 units/mL thrombin, incubating for 30 min at room temperature, and separating the serum by centrifugation in a microfuge for 5 min.

‡Serum was prepared by mixing 0.95 mL of the plasma-aprotinin mixture with 50 μL of 0.5 mg/mL BSA plus 20 units/mL thrombin and 0.5 mol/L $CaCl_2$, incubating and separating the serum as described in †.

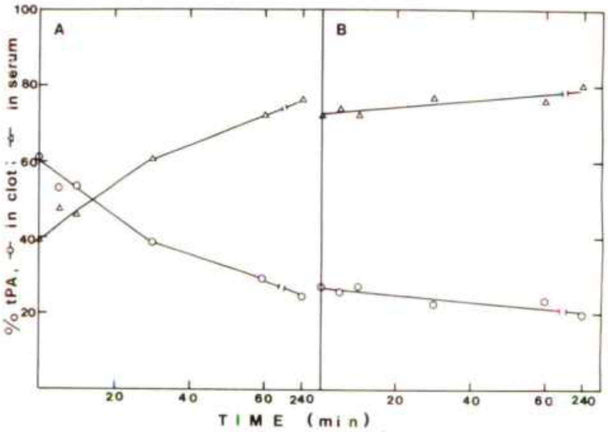

Fig 3. Effect of preclotting incubation on the partitioning of tPA antigen between clot and serum in plasma reconstituted with free tPA or tPA complex. A tPA-depleted plasma (10.8 mL, adjusted to pH 7.4) was supplemented with 1.2 mL of 10,000 U/mL aprotinin and chilled on ice for two minutes. The supplemented plasma was divided into two 6-mL aliquots. Free tPA was added to one aliquot and the other with tPA complex, such that the final concentration of tPA antigen was 17.8 and 13.6 ng/mL respectively. At 0, 5, 10, 30, 60 and 240 minutes of incubation at room temperature, 0.76 mL aliquots of each mixture was removed and clotted by addition of 40 μL of 20 U/mL thrombin, 0.5 mg/mL BSA. After 1.5 hours incubation at room temperature, the clots were packed down by vortexing and centrifugation for five minutes in a microfuge. The supernatant sera were taken for immunoradiometric quantitation of tPA antigen. Separate 0.76 mL aliquots of the mixtures were also taken, mixed with 40 μL of 0.5 mg/mL BSA and processed the same way to quantitate tPA antigen in the unclotted plasma. The % tPA antigen in the sera and clots were calculated by the following equations:

% tPA antigen in the serum =

$$\frac{[\text{tPA antigen}]\text{ in the serum}}{[\text{tPA antigen}]\text{ in unclotted plasma}} \times 100;$$

% tPA antigen in the clot = 100% − % tPA antigen in the serum.

(A) Effect of preclotting incubation on the distribution of tPA antigen between clot and serum in plasma reconstituted with free tPA. (B) Effect of preclotting incubation on the distribution of tPA antigen between clot and serum in plasma reconstituted with tPA complex.

with the time of incubation prior to clotting (Fig 3A). Reconstitution followed by immediate clotting leads to high incorporation of tPA into the clot (~60%). Incubation of the mixture for various time intervals (five minutes to four hours) before clotting results in progressively decreased partitioning of tPA antigen into the clot. At four hours incubation, only 24% tPA antigen is bound to the clot (3A), which is similar to what was found by reconstitution of tPA complex into tPA deficient plasma (Fig 3B). The time dependence of the partitioning of tPA (Fig 3) correlates well with the time course of the formation of 100-kDa tPA complex (Fig 2). Together these results suggest that free tPA possesses high affinity for a plasma clot, and that incubation of free tPA in plasma in the absence of clotting leads to the formation of a tPA complex that possesses low affinity for the plasma clot. Furthermore, since free tPA retains high affinity for fibrin upon clotting, one must assume that fibrin-bound tPA is capable of evading complexation, at least to some extent. Assuming the time course of the change in partitioning exactly reflects that of the complex formation, then the half-time for complex formation under these conditions is approximately 30 minutes.

Effect of tPA Antigen Depletion on Plasma Fibrinolytic Activity

By acidification of a plasma to pH 6 and immunoadsorption with anti-tPA-Ig Sepharose 4B, more than 98% of tPA antigen can be depleted (tPA antigen decreases from 15.4 to 0.27 ng/mL as measured by immunoradiometric assay). Using the spontaneous clot lysis assay we found that the original plasma sample lyses its clot within two days while the tPA-depleted plasma possesses no significant fibrinolytic activity (Fig 4). This result is consistent with our previous finding that spontaneous fibrinolysis in human plasma is initiated by tPA-related protein.[15]

Free tPA-Induced Plasma Fibrinolysis—Effect of Preclotting Incubation

Since depletion of plasma tPA antigen results in total loss of plasma fibrinolytic activity (Fig 4), one expects reconstitution of tPA into tPA-depleted plasma to restore fibrinolytic activity. However, because addition of free tPA to unclotted tPA-depleted plasma results in a gradual transformation of free tPA into 100-kDa complex (Fig 2) and loss of affinity of tPA antigen for fibrin upon clotting (Fig 3), we have explored whether or not this change is accompanied by alteration in fibrinolytic property. As shown in Fig 5, when free tPA was added to a tPA-depleted plasma (10 ng/mL) and followed by immediate clotting, the clot lysed within 20 hours. However, when the same supplemented plasma was allowed to stand for 10, 30, and 60 minutes at room temperature before clotting, subsequent clot lysis was progressively retarded. Figure 6 shows the effect of preclotting incubation on a plasma supplemented with various amounts of free tPA (Fig 6, A to D: 2.5, 5.0, 12.5 and 25 ng/mL, respectively). Mixing free tPA with plasma followed by immediate clotting invariably shows much faster clot lysis (open circles) than those subjected to one hour preclotting incubation (closed circles). The retardation of fibrinolysis by preclotting incubation is largely complete at low tPA concentrations (2.5 to 5.0 ng/mL) but the effect was significantly abolished at higher tPA concentration (25 ng/mL), indicating the capability of tPA-depleted plasma to inhibit tPA activity. The results from Figs 5 and 6 also suggest that fibrin formation at the time of mixing free tPA with plasma renders tPA activity stable, presumably because fibrin-bound tPA is

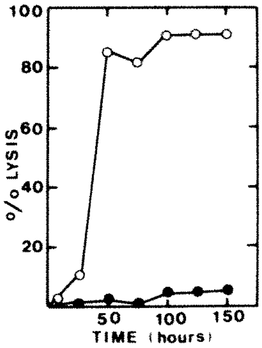

Fig 4. Effect of immunoadsorption with anti-tPA-Ig Sepharose 4B on the spontaneous fibrinolytic activity of plasma. Depletion of plasma tPA antigen by immunoadsorption with anti-tPA-Ig Sepharose 4B and assay of spontaneous plasma clot lysis were carried out as described in the experimental section. Time course of clot lysis in an untreated plasma (○). Time course of clot lysis of the same plasma depleted of tPA antigen (●).

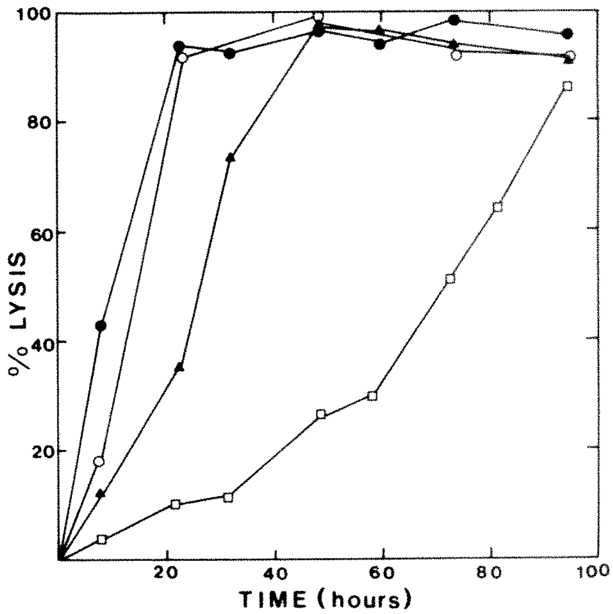

Fig 5. Effect of preclotting incubation on clot lysis in tPA-depleted plasma reconstituted with free tPA. A solution of tPA (33 μL) was added to 3.5 mL of tPA-depleted plasma, such that the final concentration of tPA was 10 ng/mL. Eight 95 μL aliquots were immediately taken into tubes and each was clotted by addition of 5 μL of 20 units/mL thrombin plus 0.5 mg/mL BSA and incubated at room temperature for 30 minutes. At 10, 30, and 60 minutes after mixing tPA and tPA-depleted plasma, eight 95 μL aliquots were again taken, clotted, and incubated as before. Clot lysis assay was performed as described in the experimental procedure. Immediate clotting (●); 10 minute preclotting incubation (○); 30 minute preclotting incubation (▲); 60 minute preclotting incubation (□).

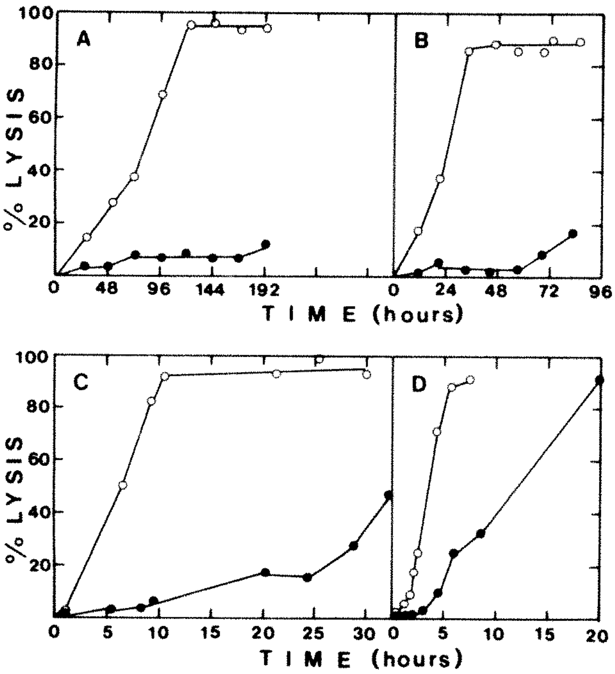

Fig 6. Induction of fibrinolysis in tPA-depleted plasma by various amounts of free tPA and the effect of preclotting incubation. Four 2-mL aliquots of tPA-depleted plasma were each supplemented with 5 μL of free tPA solution, such that the final concentrations of tPA antigen were: (A) 2.5 ng/mL; (B) 5.0 ng/mL; (C) 12.5 ng/mL; and (D) 25.0 ng/mL, respectively. Half of each mixture was immediately aliquoted into 95 μL and clotted by mixing with 5 μL of 20 units/mL thrombin plus 0.5 mg/mL BSA. The other halves were incubated at room temperature for one hour before aliquoting and clotting as before. Clot lysis assay was carried out as described in the experimental section. In each panel (A to D), (○) represents plasma clotted immediately after addition of tPA and (●) represents plasma mixed with tPA for one hour at room temperature before clotting.

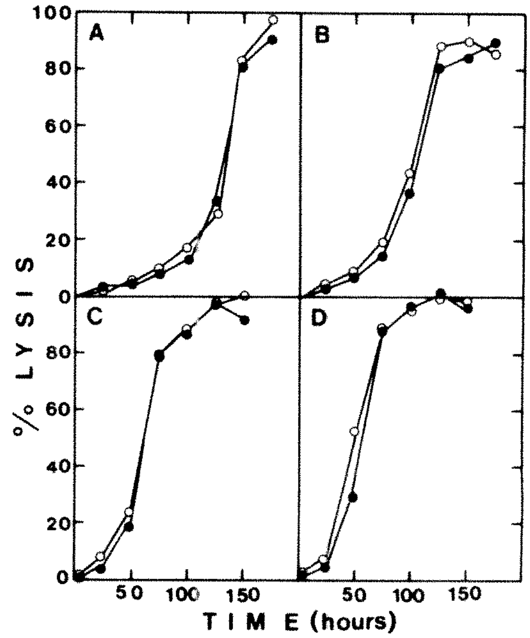

Fig 7. Induction of fibrinolysis in tPA-depleted plasma by various amounts of tPA complex and the effect of preclotting incubation. Four 1.8 mL aliquots of tPA-depleted plasma were each supplemented with 0.2 mL of tPA complex (dissolved in 0.07 mol/L Tris/0.1 mol/L glycine, pH 7.6) such that the final concentrations of tPA antigen were: (A) 12 ng/mL; (B) 18 ng/mL; (C) 29 ng/mL; and (D) 41 ng/mL, respectively. Half of each mixture was immediately aliquoted into 95 μL and clotted by mixing with 5 μL of 20 units/mL thrombin plus 0.5 mg/mL BSA. The other half was incubated at room temperature for one hour before aliquoting and clotting as before. Clot lysis assay was carried out as described in the experimental procedure. In each panel (A to D), (○) represents plasma clotted immediately after addition of tPA complex; (●) represents plasma mixed with tPA complex for one hour at room temperature before clotting.

relatively inaccessible to complexation by putative plasma inhibitors.

tPA Complex-Induced Fibrinolysis—Comparison With Free tPA-Induced Fibrinolysis

We have previously demonstrated that essentially all the tPA antigen exists in complexed form in stored plasmas.[15] The plasmas, however, still possess low-grade but strictly tPA-dependent fibrinolytic activity when clotted.[15] In order to understand better the tPA complex–induced fibrinolysis, we carried out a series of reconstitution experiments. When increasing amounts of immunoaffinity-isolated tPA complex (12 to 41 ng/mL tPA antigen) was reconstituted into tPA-depleted plasma followed by immediate clotting or incubation for one hour before clotting, progressively faster clot lysis is observed (Fig 7). In contrast to free tPA-induced clot lysis, the tPA complex-induced fibrinolysis does not show a different rate of fibrinolysis with (closed circles) or without (open circles) preclotting incubation (Fig 7). These results suggest that tPA complex-induced fibrinolysis is due either to the intrinsic activity of the complex or to the dissociation of the complex to free tPA during incubation but not to contaminating free tPA in the complex preparation. To induce similar rates of fibrinolysis, the amount of tPA complex needed is significantly higher than free tPA. Figure 8 shows the rates of fibrinolysis in relation to the concentration of free tPA or tPA complex. By plotting the rate of clot lysis ($1/S_{50}$, the inverse of the time required for 50% clot lysis) versus the concentration of added antigen, linear relationships are found. From the slopes, rate of $113 \times 10^{-4}\ h^{-1}\ ng^{-1}$ for free tPA and $5.5 \times 10^{-4}\ h^{-1}\ ng^{-1}$ for tPA complex are calculated. Therefore, free tPA is about 20-fold more active than tPA complex. The relative rates of fibrinolysis induced by isolated free tPA and tPA complex shown here may not be exactly the same as those induced by native molecules since we cannot exclude the possibility that tPA complex may be partially denatured by immunoaffinity purification. However, the result is consistent with our previous observation that stored plasma containing native tPA complex possesses relatively little activity compared to free tPA-induced fibrinolysis.[15,16]

Dissociation of tPA Complex

The immunoaffinity-isolated tPA complex does not appreciably dissociate during storage in 0.1 mol/L glycine/HCl pH 2.2 or SDS-gel electrophoresis, and plasminogen activa-

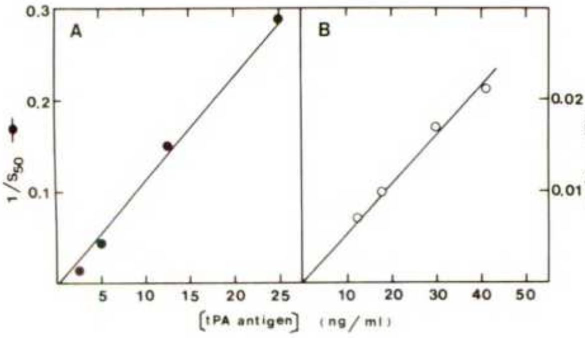

Fig 8. Relationship between rates of clot lysis and the concentration of exogenously added free tPA or tPA complex. The data were based on the experiments described in Figs 6 and 7 (without preclotting incubation). The rate of clot lysis, $1/S_{50}$, was taken as the inverse of the time required for 50% clot lysis. The concentration of tPA was calculated based on immunoradiometric assay. (A), tPA-depleted plasma supplemented with free tPA; (B), tPA-depleted plasma supplemented with tPA complex.

tor activity appears at a position corresponding to 100 kDa in zymography (Fig 9, lane 1). To investigate whether the activity may arise from dissociation of the complex, we have examined conditions under which conversion of tPA complex to free tPA occurs. Treatment of tPA complex with SDS and subsequently with Triton X-100 results in the generation of free tPA (lane 2). Furthermore, when a 100-kDa activity is cut out and eluted from a zymographic gel and rezymographed, the activity is found at 60 kDa corresponding to free tPA (lane 3). These experiments indicate that SDS and Triton X-100 treatment as occurs with zymography results in dissociation of the complex. Thus, the 100-kDa activity may be due to the dissociated free tPA. Hydroxylamine is also capable of generating free tPA from tPA complex (lane 4) and the conversion also seems to be facilitated by SDS (lane 5).

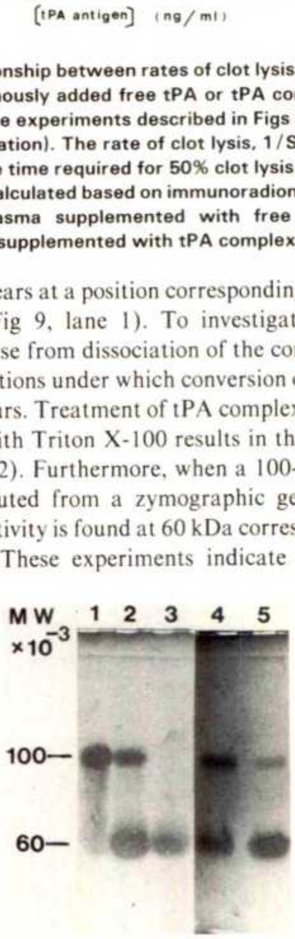

Fig 9. Zymographic study of the dissociation of tPA complex. Plasma tPA complex was isolated by immunoaffinity chromatography as described in experimental procedure and stored at −20°C in 0.1 mol/L glycine/HCl, pH 2.2. Lane 1, plasma tPA complex (8 μL) plus 2 μL 0.4 mol/L Tris/OH incubated at 37°C for 10 minutes, supplemented with 2 μL of PBB and further incubated at 37°C for 15 minutes; lane 2, plasma tPA complex (8 μL) plus 2 μL 0.4 mol/L Tris/OH containing 1% SDS, incubated at 37°C for 10 minutes, supplemented with 2 μL PBB and 1 μL 20% Triton X-100 and further incubated at 37°C for 15 minutes; lane 3, plasma tPA complex was zymographed in multiple lanes until 100-kDa activity appears as shown in lane 1. Three 100-kDa bands were cut out of the gel, extracted with 0.5 mL of 50 mmol/L NH_4HCO_3 at 37°C overnight. The extract was lyophilized and redissolved in electrophoresis sample buffer and re-zymographed; lane 4, plasma tPA complex (8 μL) plus 2 μL 0.4 mol/L Tris/OH and 2 μL 4 mol/L hydroxylamine, pH 7.0, incubated at 37°C for one hour; lane 5, plasma tPA complex (8 μL) plus 2 μL 0.4 mol/L Tris/OH containing 1% SDS and 2 μL 4 mol/L hydroxylamine, pH 7.0, incubated at 37°C for one hour.

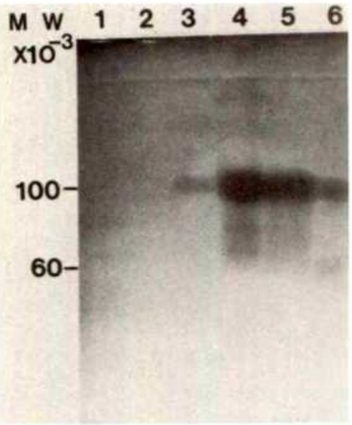

Fig 10. Immunoprecipitation of tPA-inhibitor complex from human plasma. Aliquots (0.9 mL) of pooled human plasma were mixed with 0.3 mL of 2 mol/L L-lysine and 6 μL of normal rabbit serum or various rabbit anti-PA inhibitor sera in microfuge tubes and incubated at 4°C for 12 hours. Then, 60 μL aliquots of goat antirabbit Ig (1 mL precipitates 1.5 mg rabbit Ig [Sigma product]) were added to each mixture and the incubation continued for 12 hours at 4°C to precipitate the immunocomplexes. The precipitates were collected by centrifugation for 15 minutes at 4°C in a microfuge and washed once with 1 mL PBS. The washings were discarded and the precipitates were each dissolved in 60 μL of a buffer containing 0.1 mol/L glycine/HCl, pH 2.2, 0.1% SDS, 6 mol/L urea and 0.001% bromophenol blue. Twenty μL of the redissolved precipitates were taken for SDS-PAGE (7.5% polyacrylamide) and zymography. Immunoprecipitation with: normal rabbit serum (lane 1); rabbit antihuman placental PAI-serum (lane 2); rabbit antibovine endothelial cell PAI serum (lane 3); rabbit antihuman Hep G2 PAI serum (lane 4); rabbit antihuman HT-1080 PAI serum (lane 5); rabbit antihuman HeLa tPA serum (lane 6).

Immunoprecipitation of tPA-Inhibitor Complex From Human Plasma

We have previously isolated a PA inhibitor from human placenta (designated as PAI-2*).[14] More recently we have purified PA inhibitors[20] from a human hepatoma cell, Hep G2, and from a human fibrosarcoma cell, HT-1080 (designated as PAI-1*). In order to establish the immunological relationship of the plasma tPA inhibitor with these inhibitors and an inhibitor produced by a bovine aortic endothelial cell,[21] we have carried out an immunoprecipitation experiment. As shown in Fig 10, normal rabbit serum and antiserum against placenta inhibitor do not precipitate any detectable amount of tPA-inhibitor complex (lanes 1 and 2). Antiserum against bovine aortic endothelial cell PA inhibitor precipitated a small amount of 100-kDa tPA-inhibitor activity (lane 3). Antisera against Hep G2 and HT-1080 inhibitors precipitated much larger amounts of 100-kDa tPA-inhibitor activity (lanes 4 and 5). Antiserum against HeLa cell tPA was found to precipitate the 100-kDa activity and a trace of free tPA (lane 6). The relative intensities in zymography of the immunoprecipitates are highly reproducible in several repeated experiments. The weak precipitation of 100-kDa activity by antiserum against bovine aortic endothelial cell inhibitor may be due to weaker cross-species reactivity and/or lower titer. The lower 100-kDa activity precipitated by antiserum against tPA as compared to that precipitated by antisera against Hep G2 and HT-1080 inhibitor is possibly due to the denaturing effect of antibody on tPA molecule rather than to lower titer, since the antiserum against tPA has the highest titer of these sera. The result shown in Fig 9 indicates that the plasma tPA inhibitor is immunologically related to inhibitors produced by Hep G2 cell, HT-1080 cell, and possibly also endothelial cell.

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*The term PAI-1 refers to the plasminogen activator inhibitor of endothelial cells, platelets, Hep G2, and HT-1080 cells, while PAI-2 refers to the plasminogen activator inhibitor of placenta, monocytes, and U-937 cells, in accordance with the recommendations adopted at the 32nd Annual Meeting of the International Committee on Thrombosis and Haemostasis, June 8, 1986.

DISCUSSION

We have previously demonstrated[15] that plasma fibrinolysis in vitro is dependent on the presence of tPA antigen but not on other reported plasma activators. However, it was found that essentially all the tPA antigen in the stored plasma exists in complexed form. By zymography, the major species is a 100-kDa complex. The plasma component that forms 100-kDa complex with tPA exists in slight excess over tPA in normal plasma. In this report we have further documented that the formation of 100-kDa complex is kinetically correlated with the decreased partitioning of tPA antigen into fibrin clot and depressed plasma fibrinolytic activity, suggesting that tPA-dependent activity is susceptible to inhibition in the absence of fibrin, and fibrin formation renders tPA relatively inaccessible to inhibition by plasma inhibitor. Moreover, we have demonstrated that physiological concentrations of free tPA readily induce fibrinolysis in plasma containing normal concentrations of α_2-antiplasmin. These results support a unified concept that plasma fibrinolysis is efficiently initiated and regulated at tPA level under physiological condition.

Inhibition of tPA by human plasma has been studied intensely in recent years. Earlier studies using nonphysiologically high concentrations of tPA suggested that α_2-antiplasmin and α_2-macroglobulin inhibit tPA slowly and incompletely.[22,23] More recent studies using lower concentration of tPA have found evidence for the existence of a "fast-acting inhibitor" that appears to inhibit tPA quickly (within a few minutes) and that exists in low concentration (ng/mL range) in plasma.[24-28] Using zymographic technique, incubation of tPA (100 ng/mL) in plasma leads to formation of not only the 100-kDa complex but also the 140-kDa (tPA-α_2-antiplasmin) and the 170-kDa (tPA-C1-inactivator) complexes.[25,28] These complexes were also found in plasma collected from normal subjects stimulated by 1-desamino-8-D-arginine vasopression, which greatly enhance the level of circulating tPA.[28] In contrast, our study showed that normal plasma appears to contain only one major tPA-inhibitor complex (100 kDa).[15,16] Furthermore, incubation of up to 10 ng/mL tPA in plasma for two hours does not lead to appearance of 140-kDa and 170-kDa complexes; the later complexes appear when tPA concentration is increased to 50 ng/mL (Fig 1). These results suggest that formation of 100-kDa complex is highly specific under physiological conditions in plasma and α_2-antiplasmin and C1-inactivator may participate in tPA inhibition to a significant extent only when tPA level is elevated above normal.

The concentration of the plasma component which forms 100-kDa complex with tPA is typically on the order of 5 to 10 ng/mL in normal plasma as revealed by experiments described in Fig 6. Direct isolation of this component from plasma poses a difficult challenge. An alternative approach to reveal the identity of this inhibitor is to compare it with inhibitors isolated from other sources. In our immunoprecipitation study, we have shown that the plasma inhibitor is immunologically related to Hep G2 and HT-1080 inhibitors and possibly also to endothelial cell inhibitor (Fig 10). Our preliminary comparison of the inhibitors from these cells has shown a number of differences, however. First, although the Hep G2 and HT-1080 are immunologically related, they are not identical (ie, a "spur" is found in double immunodiffusion). Second, despite that Hep G2 and HT-1080 inhibitors are isolated by the same method, the Hep G2 inhibitor is fully active but the HT-1080 inhibitor is only partially active (specific activity of HT-1080 inhibitor is ~20% that of Hep G2 inhibitor). Third, the activity of isolated Hep G2 inhibitor is decreased after SDS-treatment while that of HT-1080 inhibitor is enhanced. Based on the latency property and SDS-stimulating effect, the HT-1080 inhibitor (but not the Hep G2 inhibitor) is similar to the endothelial inhibitor.[21,29] Because of these incompletely resolved questions, more work is required to establish an unambiguous relationship among these cellular and plasma inhibitors.

Previous studies by other investigators[24,25,27,28] have used higher concentrations of free tPA (63 to 180 ng/mL) to study tPA inhibition in plasma. In inhibitor-rich plasma from patients (containing up to 87 ng/mL inhibitor) inhibition was found to be biphasic—a fast phase (minutes) followed by a slow phase (hours). In normal plasma, inhibition occurred in a single slow phase (hours). The fast phase could be attributed to the interaction with plasma inhibitor that forms 100-kDa complex with tPA; the slow phase reaction was possibly due to the formation of tPA-α_2 antiplasmin, tPA-C1 inactivator (C1INA), and 100-kDa complexes.[25,28] In our study, we have used close to physiological concentrations of free tPA (2.5 to 10 ng/mL) to study its inhibition in normal plasma. Under these conditions, the tPA inhibition occurs with a time course of one hour or more and the complex formed appears to be restricted mainly to the 100-kDa complex as shown in zymography. These results suggest that under physiological conditions (low free tPA and inhibitor concentrations), the tPA inhibition through 100-kDa complex formation occurs rather specifically and the process is actually not "fast." Theoretically, if one assumes that the reaction follows: $E + I \xrightleftharpoons{k_1} EI \rightleftharpoons EI'$, where E is the enzyme, I the inhibitor, I′ the modified inhibitor, and the second order rate constant, $k_1 = 5 \times 10^6$ mol/L^{-1} · S^{-1} (k_1 was estimated to be 1×10^6 mol/L^{-1} · S^{-1} or 1×10^7 mol/L^{-1} · S^{-1} using inhibitor rich plasma),[24-26] the t½ can be calculated to be 2.6 minutes when the initial concentrations of free tPA and inhibitor are 1.3 nmol/L (~85 ng/mL tPA). In contrast, if the initial concentrations of free tPA and inhibitor are 75 pmol/L (~5 ng/mL tPA) the t½ would be 44 minutes. Therefore, from experimental and theoretical considerations, the reaction between tPA and plasma inhibitor under physiological conditions may take more than one hour to complete because tPA and inhibitor concentrations are exceedingly low. These considerations also raise some questions concerning methods for functional assay of plasma tPA inhibitor. The assumption that the reaction is complete within a few minutes may not hold true when physiological concentrations of tPA and inhibitor are involved.

Slow release of proteinases from inhibitor complexes has been observed in other systems such as trypsin-α_1 proteinase inhibitor.[30] Our in vitro studies suggest that tPA-inhibitor complex may have some intrinsic activity or may undergo slow dissociation as well. The exact mechanism by which the activity arises remains to be determined. In analogy to other proteinase-inhibitor systems[30] one may postulate that proteinases interact with the inhibitors resulting in the formation of enzyme-modified inhibitor complexes that slowly dissociate. Alternatively, other proteinases may attack the enzyme-virgin inhibitor complexes to form enzyme-modified inhibitor complexes that subsequently undergo dissociation. At present, the significance of the preformed tPA-inhibitor complex in plasma fibrinolysis relative to free tPA-induced fibrinolysis remains unclear.

The data presented have led us to formulate the following model for the initiation and regulation of tPA-dependent fibrinolysis in circulation. We postulate that tPA is released into the circulation as free tPA. In the event of fibrin formation, free tPA is adsorbed onto fibrin, which renders tPA relatively inaccessible to complexation by putative tPA inhibitors in the plasma. The fibrin-bound tPA activates the fibrin-bound plasminogen to plasmin, which degrades the fibrin before plasmin inhibitors take effect. In the absence of fibrin, the released free tPA is subjected to gradual inhibition by the inhibitors. By this mechanism, fibrinolysis is highly fibrin-localized and efficiently regulated at PA level. Besides the above pathway, preformed tPA-inhibitor complex may also contribute to fibrinolysis to some extent by an as yet unknown mechanism.

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Engraftment of Dogs With Ia-Positive Marrow Cells Isolated by Avidin–Biotin Immunoadsorption

By Ronald J. Berenson, William I. Bensinger, Dale Kalamasz, Friedrich Schuening, H. Joachim Deeg, Theodore Graham, and Rainer Storb

Previous work has shown failure of engraftment in lethally irradiated dogs when autologous marrow was depleted of Ia-positive cells with an anti-Ia antibody and complement before infusion. In the current study, we have utilized an avidin–biotin immunoadsorption procedure to obtain a population of highly enriched Ia-positive cells for autologous bone marrow transplantation in dogs given lethal irradiation. Dog marrow cells (2.4 to 7.0 × 10^9 cells) that contained 8.6% to 19.9% Ia-positive cells were treated successively with monoclonal antibody 7.2, which reacts with a framework determinant of Ia-antigen, and biotin-conjugated goat antimouse immunoglobulin. These treated cells were passed over a column of avidin–Biogel (polyacrylamide) and the adherent cells removed by mechanical agitation. Seven lethally irradiated dogs were transplanted with 5.9 to 33.4 × 10^6 recovered adherent cells per kilogram of which 69.0% to 88.0% were Ia-positive. All dogs had hematologic recovery; six are alive and well with durable engraftment and one died on day 15 posttransplant. They are immunologically normal as determined by lymph node and bone marrow biopsies, lymphocyte function, and immunophenotyping of peripheral blood and bone marrow cells. These data provide further evidence that canine hematopoietic stem cells express Ia-like antigens and that these cells are capable of complete hematopoietic and immunologic reconstitution in an autologous model.

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THE EXPRESSION OF IA-LIKE (class II) antigens on hematopoietic progenitors has been the subject of intense study by numerous investigators. These polymorphic bimolecular glycoproteins with molecular weights of approximately 29,000 and 34,000 daltons are coded for by the D region and the I region of the major histocompatibility complex in man (HLA) and mice (H2), respectively.[1] Numerous monoclonal antibodies (McAb) recognizing human DR antigens cross-react with a similar bimolecular glycoprotein complex in the dog.[2,3] Previous in vivo studies have demonstrated failure of hematopoietic reconstitution in lethally irradiated dogs given autologous marrow depleted of Ia-positive cells with McAb 7.2, which reacts with human DR antigens, and complement.[4] These data suggest that Ia-like antigens may be present on canine hematopoietic stem cells. However, it is also possible that hematopoietic reconstitution did not occur because an Ia-positive accessory cell necessary for engraftment was eliminated. Therefore, it was desirable to perform autologous marrow transplants with Ia-positive cells to test whether pluripotent hematopoietic stem cells express Ia-like antigens in dogs.

Immunoaffinity chromatography has been used to separate the large numbers of cells required for bone marrow transplantation (BMT).[5] We have recently developed an immunoaffinity system which uses avidin-coated beads to bind cells labeled with biotin-conjugated antibodies.[6-8] The very high affinity between the protein avidin and the vitamin biotin ($K_D = 10^{-15}$ mol/L) allows cells to be separated without prolonged incubation required by immunoaffinity columns using antibodies, lectins, or protein A as the ligands. Prolonged incubation has been shown to increase the nonspecific binding of cells. To amplify the biotin, "signal" marrow cells are first labeled with a mouse McAb directed against the antigen of interest and then secondarily treated with biotin-conjugated goat antimouse antibody. Antibody-treated cells are passed over a column of avidin-coated beads that will bind antibody-labeled cells and allow nonlabeled cells to pass through the column. Previous work has shown that the bound cells recovered by mechanical agitation are a relatively pure cell population that retains normal in vitro cellular functions.[7] We have used this procedure to isolate a population of highly enriched Ia-positive cells from canine marrow and performed successful autologous marrow transplants by infusing these cells into lethally irradiated dogs.

MATERIALS AND METHODS

Dogs

Beagles, hounds, and mongrels from the Fred Hutchinson Cancer Research Center kennels were dewormed, vaccinated against distemper, leptospirosis, hepatitis, and parvovirus, housed in single cages, and observed for disease for at least two months. The dogs weighed 6.7 to 22.5 (median 15.0) kg and their ages were 9 months to 2+ years old at the time of transplantation. Research was conducted according to the principles enunciated in the "Guide for Laboratory Animal Facilities and Care" prepared by the National Academy of Sciences–National Research Council. Dogs were observed for 2 to 6 months post-BMT.

Total Body Irradiation, Marrow Infusion, and Animal Care

Bone marrow was obtained from anesthetized dogs as previously described.[9] After marrow aspiration, dogs were given 9.2 Gy of total body irradiation at 7 cGy/min from two opposing ^{60}Co sources.[10,11]

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The positively selected (Ia-positive) marrow cells were infused within two to four hours of irradiation. Postgrafting parenteral fluids, electrolytes, platelet transfusions, and antibiotics were administered as indicated. All blood products used for transfusion were irradiated in vitro (15 Gy) to inactivate pluripotent stem cells and immunologically competent cells. Hematocrit, leukocyte, and platelet counts were obtained daily and an autopsy with histological examination was performed on the dog that died.

Antibodies

A purified murine monoclonal IgG2b antibody 7.2 was kindly provided by Dr John Hansen. McAb 7.2 reacts with human HLA-DR antigens and cross-reacts with similar Ia-like antigens (p 29/34) on canine cells.[2,3] Biotinylated goat antimouse immunoglobulin (B-GMAIg), fluorescein isothiocyanate conjugate (FITC) of swine antigoat immunoglobulin, FITC-swine immunoglobulin, and FITC–goat antimouse immunoglobulin were obtained from Tago (Burlingame, CA). McAb 9E8 (donated by Genetic Systems, Seattle, WA) recognizes the p(15)E antigen of murine leukemia virus and is not reactive with dog or human tissues, including bone marrow and peripheral blood. Antibodies reactive with dog cells have been previously described. McAb DT2 reacts with canine helper T lymphocytes, while E11 recognizes suppressor T lymphocytes.[12,13] McAb DLY1 reacts with both lymphocytes and monocytes, while DLY6 recognizes lymphocytes.[14,15] McAb Thy1 recognizes lymphocytes and natural killer cells in dogs.[16]

Production of Avidin–Biogel

Avidin obtained from Sigma Chemical (St Louis, MO) was conjugated to Biogel P-30 (Bio-Rad, Richmond, CA) using a modification of our previously described procedure.[7] Carboxylation of Biogel P-30 was first performed by incubation of gel in 0.5 mol/L sodium carbonate and 0.5 mol/L sodium bicarbonate at pH 10.5 for two hours at 60°C. The gel was cooled, washed with phosphate buffered saline (PBS), and stored in PBS with 0.1% sodium azide at 4°C. Activation of the carboxylated gel was accomplished with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC-HCl). For avidin linkage 100 mL of carboxylated gel was incubated with 400 mg EDC-HCl (4 μg EDC-HCl/mL gel) in a total volume of 500 mL distilled water at pH 5 for five minutes at room temperature. Ten milligrams avidin (100 μg avidin/mL gel) was then directly added to this mixture and the incubation continued for two hours. The efficiency of conjugation exceeded 98% as determined by measurement of protein concentration of this solution after incubation with the gel. Glycine at a final concentration of 0.2 mol/L was added to block unconjugated sites with a two-hour incubation, followed by washing with PBS and storage with 0.1% sodium azide at 4°C. The column containing avidin–Biogel was sterilized with ethylene oxide gas sterilization. Microbiologic cultures performed on small samples of eluate before and after column use demonstrated absence of bacteria or fungi in all experiments.

Selection of Ia-positive Cells

Cell preparation. For the first transplanted dog, C239, cells were obtained by Ficoll-Hypaque (specific gravity, 1.077; Pharmacia, Piscataway, NJ) density gradient centrifugation at 900 g for 25 minutes. However, subsequent in vitro studies demonstrated less nonspecific binding when a buffy coat marrow cell preparation rather than Ficoll-Hypaque–separated marrow cells were utilized in the avidin-biotin immunoadsorption procedure.[8] All other dogs therefore had the cells obtained from a buffy coat preparation by centrifugation at 250 g for 15 minutes.

Antibody treatment and column separation. Marrow cells at a concentration of 50 × 10^6 cells/mL were incubated in PBS with 2% bovine serum albumin (PBS/BSA) with 20 μg/mL McAb 7.2 for 30 minutes at 4°C. The cells were washed twice before and after incubation at an identical cell concentration in PBS/BSA with 1:100 dilution of B-GAMIg for an additional 30 minutes at 4°C. The cells were adjusted to a concentration of 25 × 10^6 cells/mL in PBS/BSA and 1.5 to 2.0 × 10^9 cells passed over a Chromaflex (Kontes, Vineland, NJ) 15-cm × 2.5-cm column containing 25 mL avidin–Biogel at a flow rate of 20 mL/minute until a total volume of 150 to 175 mL of eluate was collected. Approximately 50 mL of PBS was passed at the same flow rate through the gel to wash out BSA. The adherent cells were dislodged by mechanical agitation with a 10-mL pipette until 150 mL total volume had been collected. After a small sample of the recovered cells was obtained for immunofluorescence studies to determine the purity of the recovered cell population, the recovered Ia-enriched cells were resuspended in 20 to 30 mL PBS and infused into the irradiated dog. Marrow preparation, antibody treatment, and column separation were carried out with sterile technique in a laminar airflow hood. The entire marrow separation procedure required approximately three hours from start to finish.

Immunofluorescence studies. The enrichment of Ia-positive cells was determined by immunofluorescence staining and FACS analysis. Briefly, 5 × 10^5 cells were treated with hemolytic buffer, washed twice, and then incubated with a 1:20 dilution of FITC–swine antigoat immunoglobulin in PBS/BSA with 0.1% sodium azide for 20 minutes at 4°C. The stained cells were washed twice and analyzed with a FACS IV and computer 440 (Becton Dickinson, Mountain View, CA). The starting and adsorbed cells labeled with McAb 7.2 and B-GAMIg were stained with FITC–swine antigoat immunoglobulin. As a negative control, the unlabeled marrow cells were stained with FITC–swine antigoat immunoglobulin or incubated successively with McAb 9E8 and B-GAMIg and stained with FITC–swine antigoat immunoglobulin. The percentage of Ia-positive cells was determined by subtracting cells labeled with the control reagents from the antibody-labeled starting and adsorbed cells. As an additional control in selected experiments, we demonstrated that neither the starting nor the adsorbed cells stained with FITC–swine immunoglobulin without antigoat reactivity. For immunophenotyping of transplanted dogs, samples of peripheral blood and bone marrow mononuclear cells were stained with the McAb (DT2, E11, 7.2, DLY1, DLY6, or Thy1) and FITC–goat antimouse immunoglobulin and then subjected to FACS analysis using established methods.[13]

Mitogenic assay and mixed lymphocyte reaction. These experiments were performed as previously described.[12,15] In the mitogenic assay, 10^5 peripheral blood mononuclear cells (PBMC) were incubated in the presence (or absence) of 20 μg/mL concanavalin A (Con-A, Calbiochem, San Diego, CA) in Waymouth's medium with 20% heat-inactivated canine serum for three days at 37°C in a 5%-CO_2-in-air incubator. In the mixed lymphocyte reaction (MLR), 10^5 PBMC were cultured with 10^5 irradiated allogeneic (or autologous) stimulator PBMC for six days at 37°C in a 5% CO_2/air atmosphere. One microcurie of ^{3}H-thymidine (Amersham, Arlington Heights, IL) was added to each sample 16 hours before harvesting. Triplicate samples were harvested onto filter paper, placed in scintillation fluid, and counted in a scintillation counter.

RESULTS

Immunofluorescence Studies

Figure 1 shows immunofluorescence staining with FITC–swine antigoat immunoglobulin and FACS analysis of marrow cells (from dog BC128) treated with McAb 7.2 and B-GAMIg before (Start) and after adsorption onto avidin–Biogel (Adsorbed). The starting cell population was 15.0% Ia-positive, while the recovered adsorbed cells given to the dog were 85.0% Ia-positive. Further studies were performed

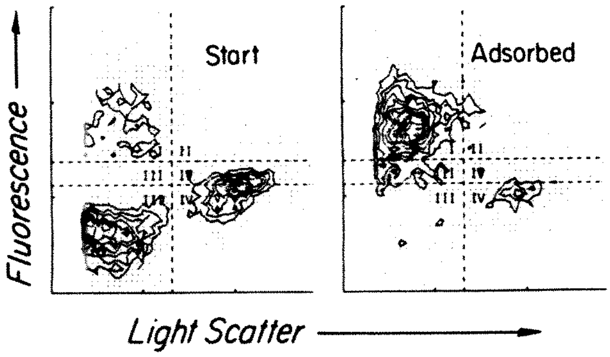

Fig 1. Bone marrow cells from dog BC128 treated successively with McAb 7.2 and B-GAMIg and passed over avidin–Biogel. Antibody-treated cells (Start) and recovered adherent cells (Adsorbed) stained with FITC–swine antigoat immunoglobulin and subjected to FACS analysis.

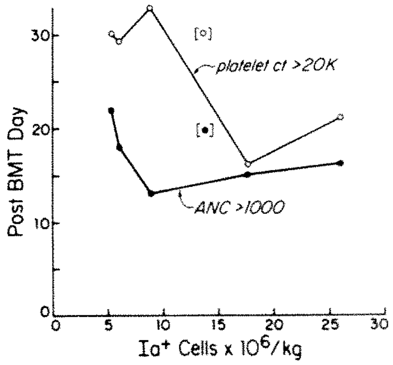

Fig 2. Rate of engraftment as measured by post-BMT day when absolute neutrophil count greater than 1,000/μL and platelet count greater than 20,000/μL compared with number of infused Ia-positive cells per kilogram. Values in brackets indicate dog receiving Ia-positive cells obtained from Ficoll-Hypaque–separated marrow.

to determine the type of cells present that were Ia-positive and Ia-negative in the recovered adsorbed cell population. FACS sorting and subsequent light microscopic examination of the adsorbed cell fraction demonstrated that the Ia-positive cells were predominantly mononuclear cells, while the nonspecifically bound Ia-negative cell fraction contained greater than 90% granulocytes.

Marrow Transplants Using Ia-positive Cells

A total of seven dogs was transplated with Ia-positive cells obtained using the avidin–biotin immunoadsorption procedure (Table 1). The first transplanted dog, C239, received cells obtained by Ficoll-Hypaque separation, treated successively with McAb 7.2 and B-GAMIg, and passed over avidin–Biogel. From a starting cell population of 2.56 × 10^9 cells of which 14.8% were Ia-positive, we recovered 2.28 × 10^8 adsorbed cells that were 69% Ia-positive. In the six other dogs, buffy coat preparations of dog marrow were obtained containing 2.4 to 7.0 × 10^9 cells that were 8.6% to 19.9% Ia-positive. These cells were treated successively with McAb 7.2 and B-GAMIg and passed over avidin–Biogel. A total of 1.15 to 6.68 × 10^8 adherent cells was recovered by mechanical agitation of which 75.0% to 88.0% were Ia-positive by immunofluorescence staining and FACS analysis. The overall recovery of the Ia-positive cells originally present in the starting cell population averaged 41.0 ± 15.6% (range: 25.0% to 62.8%). Infusion of the recovered cells into autologous recipients conditioned with 9.2 Gy of whole body irradiation has produced hematopoietic reconstitution and long-term survival in six dogs. All these dogs developed completely normal hematological parameters that remained durable until the time of sacrifice 48+ to 195+ days after transplant. A seventh dog died at day 15 of overwhelming sepsis from a former intravenous shunt site with bone marrow evidence of engraftment at autopsy.

Slight delay in engraftment of granulocytes and platelets was observed in some of the dogs compared to historical control lethally irradiated dogs receiving unseparated autologous marrow (Table 1). Figure 2 shows the relationship between the number of Ia-positive cells infused and rate of engraftment. Delay of platelet engraftment was observed when less than 15 × 10^6 Ia-positive cells/kg were given, while granulocyte engraftment was slowed when less than

Table 1. Engraftment Data

	Before Treatment		After Treatment				Post-BMT Day		
Dog	Total Cells* × 10^8	% Ia$^+$	Total Cells × 10^8	Total Cells × 10^6/kg	% Ia$^+$	Ia$^+$ Cells × 10^6/kg	ANC† $>10^3/\mu L$	Platelets‡ $>20 \times 10^3/\mu L$	Survival§ (days)
C239	25.6	14.8	2.28	19.9	69.0	13.7	20	30	65+
BB259	26.5	8.6	1.86	23.2	75.0	17.4	15	16	48+
BC125	24.0	11.2	1.93	9.9	87.5	8.7	13	33	63+
C234	29.4	18.0	2.22	19.0	77.0	14.6	—	—	15
C240	70.0	19.9	6.68	33.4	77.3	25.8	16	21	53+
BC126	25.0	15.0	1.16	5.9	88.0	5.2	22	30	195+
BC128	27.1	14.5	1.15	7.2	85.0	6.1	18	29	178+
Normal‖ Unseparated (4.0 × 10^8/kg)				—	—	—	12	15	
(n = 16)							(8-14)	(11-23)	

*Cells obtained from buffy coat preparation except dog C239, which received Ficoll-Hypaque separated cells.

†Day after transplant when absolute neutrophil count greater than $10^3/\mu L$.

‡Day after transplant when platelet count greater than 20 × $10^3/\mu L$.

§Dogs were sacrificed (except dog C234) at day post-BMT listed; dog C234 died at day 15 post-BMT from sepsis with bone marrow at autopsy showing trilineage engraftment.

‖Historical control dogs that were transplanted with a median of 4.0 × 10^8 marrow cells/kg with median and range of values for neutrophil and platelet engraftment.

Table 2. Mitogenic Response to Con-A of PBMC from Transplanted Dogs BC126 and BC128*

	Media	Con-A	Stimulation Index
BC126	506 ± 197	12,613 ± 512	24.9
BC128	256 ± 140	24,294 ± 1,766	94.9
Control	163 ± 60	6,539 ± 939	40.1

*PBMC from transplanted dogs BC126 and BC128 as well as a normal (control) dog were incubated in the presence or absence of Con-A (see Materials and Methods). Mean CPM of triplicate cultures are shown. A stimulation index was calculated as follows: cpm Con-A–stimulated PBMC/cpm media-incubated PBMC.

10 × 10^6 Ia-positive cells were infused into dogs. However, the dogs who received lower numbers of Ia-positive cells also received less total cells, which may in itself be responsible for slow engraftment. The single dog receiving Ficoll-Hypaque–separated marrow cells appeared to have more delayed engraftment than the other dogs receiving comparable numbers of buffy coat separated cells.

Posttransplant Analyses of Dogs BC126 and BC128

Histopathological studies and immunologic studies were performed on dogs BC126 and BC128 approximately 3 to 4 months after BMT. Lymph node and bone marrow biopsies were normal in these dogs (data not shown). Normal T lymphocyte function was demonstrated by both in vitro and in vivo studies. Proliferative responses to the T lymphocyte mitogen, Con-A, were excellent (Table 2). Normal stimulation of peripheral lymphocytes by irradiated allogeneic cells in MLR was documented (Table 3). In vivo studies showed that the two dogs rejected skin grafts from allogeneic donors in 12 days (normal range: 11 to 14 days). Immunophenotyping of peripheral blood and bone marrow revealed normal percentages of cells reactive with McAbs 7.2, DT2, E11, DLY1, DLY6, and Thy1 (data not shown).

DISCUSSION

We have previously shown with negative selection that canine stem cells express Ia antigens. In that study, dogs given lethal total body irradiation (9.2 Gy) and an autologous marrow depleted of Ia-positive cells with McAb 7.2 and complement failed to engraft.[4] In contrast, Prendergast et al observed slow marrow recovery over a 2-month period in irradiated dogs receiving autologous marrow depleted of Ia-positive cells.[17] However, the dogs were treated with lower doses of radiation (5 Gy), suggesting that the slow hematologic improvement may be from endogenous recovery of host marrow rather than the Ia-depleted infused marrow.

Table 3. Mixed Lymphocyte Reaction of PBMC from Transplanted Dogs BC126 and BC128*

Dog	Autologous	Allogeneic	Stimulation Index
BC126	149 ± 78	3,948 ± 687	26.6
Control	238 ± 74	4,037 ± 995	17.0
BC128	774 ± 54	5,011 ± 912	6.5
Control	602 ± 134	10,320 ± 1,082	17.1

*PBMC from transplanted dogs BC126 and BC128 as well as from two normal (control) dogs cultured with autologous or irradiated allogeneic PBMC (see Materials and Methods). Mean cpm of triplicate cultures are shown. A stimulation index was calculated as follows: cpm allogeneic stimulated PBMC/cpm autologous stimulated PBMC.

Because of the continued controversy regarding the expression of Ia-antigens on stem cells, we wished to conduct a study in which positively selected Ia-positive cells were transplanted into lethally irradiated dogs. Workers have previously been unable to perform this experiment because of the lack of a method to separate the large number of cells required for BMT. Although FACS sorting can provide relatively pure cell populations, its limited throughput makes it impractical for large-scale application.[18] The "panning" technique, in which cells are separated on plastic petri dishes coated with antibodies, produces variable yield and purity and is cumbersome to use for separating large numbers of cells.[19]

In the current study, we used avidin–biotin immunoadsorption chromatography to purify Ia-positive cells using the same antibody (7.2) as previously employed for negative selection. This highly enriched population of Ia-positive cells was infused into seven lethally irradiated dogs. Sustained and complete marrow engraftment was observed in six out of seven transplanted dogs, while a seventh dog died from sepsis at day 15 post-BMT with histologic evidence of engraftment. Detailed studies of two dogs also documented recovery of normal immunologic function after transplanting Ia-positive cells. These data suggest that marrow stem cells responsible for hematologic and immunologic reconstitution express Ia antigens.

Although dogs were transplanted with predominantly Ia-positive cells, it was also possible that contaminating Ia-negative cells infused into these dogs could be responsible for marrow engraftment. FACS sorting experiments of the recovered adherent cells showed that the nonspecifically bound Ia-negative cells consisted almost entirely of granulocytes. It thus seems unlikely that these infused Ia-negative cells represent pluripotent hematopoietic stem cells. However, we cannot exclude the possibility that a subpopulation of the infused cells (Ia-positive or Ia-negative) played an accessory role in marrow engraftment.

In humans, Ia-like antigens are found on committed hematopoietic progenitors cultured in semisolid medium, including early erythroid progenitor cells (BFU-E), late erythroid progenitor cells (CFU-E), granulocyte-macrophage progenitor cells (CFU-GM), and multipotent progenitor cells giving rise to megakaryocytes as well as erythrocyte, granulocyte, and macrophage progenitor cells (CFU-GEMM).[20-24] More recently, it has become apparent that at least three subloci (HLA-DP, HLA-DQ, HLA-DR) exist within the HLA-D region.[25-27] Recent studies have detected some of these antigens (HLA-DP and HLA-DR) on both committed progenitors and potentially more primitive cells capable of sustaining marrow growth in long-term culture.[28-30] Similar results have also been reported in the mouse using the CFU-S assay with I-E antigens (equivalent to human HLA-DR) detected on CFU-S and I-A antigens (equivalent to human HLA-DQ) lacking on these cells.[23]

The application of our results to human (or murine) hematopoietic stem cells must be done with caution.

Although McAb 7.2 has been shown to react with a similar bimolecular glycoprotein complex on both human and dog cells, the DLA-D region has not been well-characterized in dogs. Furthermore, the distribution of class II antigens in dogs appears to differ somewhat from that reported for humans. Human Ia-antigens have been detected on B lymphocytes, monocytes, and activated T lymphocytes, while unstimulated as well as activated T lymphocytes, in addition to B lymphocytes and monocytes, express Ia-antigens in dogs.[1,2] Despite these problems, the data in mice, humans, monkeys, and dogs are all consistent with the hypothesis that hematopoietic stem cells express class II antigens. It may be possible to use anti-Ia antibodies to specifically isolate hematopoietic stem cells for human BMT.

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Receptor-Specific Inhibition of Bone Marrow Erythropoiesis by Recombinant DNA-Derived Interleukin-2

By Stefan E.G. Burdach and Lee J. Levitt

Interleukin-2 (IL-2) induces differential secretion of lymphokines by IL-2 receptor (IL-2R)-positive and IL-2R-negative T cells. We studied T cell IL-2R-specific modulation of adult bone marrow erythropoiesis by recombinant IL-2 (rIL-2). IL-2R were induced by CD3 T cell surface determinant-triggering and analyzed by cytofluorography. Bone marrow monocyte and T cell-depleted (NAB-T) target cells were assessed for early erythroid progenitor expression (BFU-E) in the presence of 0 to 10^3 U/mL of rIL-2. rIL-2 had no significant effect on BFU-E expression in the absence of T cells or in the presence of IL-2R-negative T cells. rIL-2 caused a dose-dependent inhibition (75% to 90%) of BFU-E in the presence of autologous IL-2R-positive T cells. The addition of anti-IL2-receptor antibody to cultures containing rIL-2 plus IL-2R-positive T cells entirely abrogated rIL-2-mediated inhibition of BFU-E. In the presence of rIL-2 (10^2 U/mL) production of interferon gamma (IF-gamma) by adult marrow CD3-triggered IL-2R-positive T cells was increased 37- to 125-fold compared to IL-2R-negative T cells. rIF-gamma caused a dose-dependent (88% ± 17% at 10^3 U/mL) inhibition of adult BFU-E in the presence of CD3-triggered autologous T cells. rIL2-mediated inhibition of adult BFU-E in the presence of IL-2R-positive T cells was partially abrogated (52% ± 16%) following addition of monospecific IF-gamma antibody. These results demonstrate (a) rIL-2 modulation of adult marrow erythropoiesis is selectively dependent upon both the presence or absence of autologous T cells and the IL-2R status of these T cells; and (b) rIL-2-induced inhibition of adult marrow erythropoiesis is mediated in part by release of IF-gamma from IL-2R-positive T cells.

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EXPERIMENTAL EVIDENCE indicates that T lymphocytes and specific T-derived lymphokines play an important and complex role in the regulation of erythropoiesis.[1-8] The concept that lymphokines as identifiable proteins mediate specific regulatory functions provides a relevant approach for clarifying the physiology of hematopoietic immunoregulation. The availability of human lymphokines produced by recombinant gene technology enables further dissection of the regulatory mechanisms involved in cellular communication between the hematopoietic and immune systems.

Interleukin-2 (IL-2) is a 15-kd glycoprotein produced by helper T lymphocytes in response to both antigen presentation and stimulation with macrophage-derived interleukin-1.[9,10] IL-2 induces proliferation of both helper/inducer and suppressor/cytotoxic T cells with accompanying lymphokine release.[9,10] Human IL-2 has been biochemically characterized and molecularly cloned and is known to be encoded by a single gene located on chromosome 4.[11] Recombinant DNA-derived IL-2 (rIL-2) has recently been made available as a purified protein.[12]

It has been established by several investigators that the response of T cells to IL-2 requires expression on the T cell surface of the 55-kd IL-2 receptor.[9,10,13] Two monoclonal antibodies recognizing the same determinant have been raised against this IL-2 receptor (IL-2R) and have been termed anti-Tac-1[14,15] and 2A3.[16] This IL-2 receptor is induced by antigen presentation to the T cell receptor for antigen (T_3/T_i complex).[9,17-19] Presentation of antigen to the T cell antigen receptor can be substituted by binding of anti-T_3 (CD3) monoclonal antibody[19] or lectins.[20] Following stimulation with antigen or lectin, T cells primarily express a low-density IL-2R.[19,21] This receptor has a high affinity for IL-2 and has been thought to be responsible for mediating biologic effects of the lymphokine.[21] In response to IL-2, T lymphocytes increase their IL-2R expression,[21] express a low-affinity IL-2R[21] and release interferon gamma (IF-gamma).[22,23]

The precise regulatory role of IL-2 in hematopoiesis has not yet been determined. We studied modulation of adult marrow early erythroid progenitor (BFU-E) expression by IL-2R-positive and IL-2R-negative T cells in response to purified recombinant human IL-2 and purified human recombinant IF-gamma. Our results demonstrate that IL-2 exerts a specific regulatory role on erythropoiesis in vitro, that IL-2 modulation of adult marrow erythropoiesis is selectively dependent upon both the presence of autologous T cells and the IL-2 receptor status of those T cells, and that IL-2-induced inhibition of adult early erythroid progenitors in the presence of IL-2R-positive T cells is mediated at least in part by IF-gamma.

MATERIALS AND METHODS

Recombinant DNA-derived lymphokines. Highly purified human interleukin-2, derived from *E coli* transfected with human c-DNA,[12] was kindly provided by Drs Kirston Koths and Ed Bradley of Cetus Corporation, Emeryville, CA. Specific activity, as assessed by ^{3}H-thymidine incorporation into IL-2-dependent murine T cell lines, ranged from 4.2 to 6.0 × 10^9 U/g protein. The purity of the preparations was 98% by either sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) densitometry or high-performance liquid chromatography (HPLC). An excipient control

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was used as an IL-2 buffer control and contained all ingredients of the IL-2 formulation except IL-2. Human recombinant DNA-derived IF-gamma was produced in *E coli* following gene synthesis and transfection[24] (AMGen, Thousand Oaks, CA). The material was >95% pure by SDS-PAGE densitometry. The endotoxin content of the lymphokine stocks and working solutions was assessed by limulus assay; endotoxin content was at all times negligible ($<10^{-10}$ g/mL).

Cells and cell separation procedures. All studies were approved by the Stanford University Human Subjects Experimentation Committee. Human bone marrow was obtained from normal adult donors. The low density (buoyant) mononuclear cell fraction was depleted of monocytes by two consecutive plastic adherence procedures as previously described.[7] Residual monocyte contamination of NAB cells was assessed by indirect immunofluorescence with monoclonal antibody Leu M3 and FITC-conjugated purified goat antimouse polyclonal IgG and was <1% . The adherent fraction was recovered at 4°C from the first adherence procedure.[7] Indirect fluorescence immunophenotyping of the adherent fraction yielded 62% to 77% of the cells positive with monocyte monoclonal antibody Leu M3, 12% to 34% positive with pan-B lymphocyte CD20 (B1, Coulter, Hialeah, FL) monoclonal antibody, and less than 1% positive with either pan-T lymphocyte CD3 (IgG_1 Leu 4) or pan-natural killer lymphocyte CD16 (Leu 11c) monoclonal antibodies.

The nonadherent buoyant (NAB) mononuclear cell fraction was further depleted of T cells by solid-phase indirect immunoabsorption as previously described.[7] Nonbound (NAB-T) and bound (T) cells were recovered by differential elution at 4°C and 23°C, respectively. Residual T lymphocyte contamination of NAB-T cells as assessed by indirect immunofluorescence following simultaneous incubation with anti-CD5 (Leu 1, provided by Dr R. Levy, Stanford University), anti-CD8 (Leu 2), and anti-CD4 (Leu 3) antibodies was less than 1%. Purity of the bound (T) fraction recovered from the first-step immunoabsorption procedure was 95% ± 3% as assessed by a three-stage immunofluorescence assay employing anti-CD3 IgG (Leu 4), goat antimouse IgG, and rabbit antigoat IgG-FITC (fluorescein isothiocyanate conjugate).

Induction of IL-2 receptor. Bone marrow mononuclear cells were depleted of monocytes to blunt endogenous IL-1 and subsequent IL-2 production. Abrogation of endogenous IL-2 production was monitored by IL-2 bioassay. Nonadherent cells were incubated with CD3 monoclonal antibody (OKT3 IgG_{2a}, 1.25×10^{-7} g/10^6 cells), which recognizes a determinant (CD3) on the T cell surface associated with the T cell receptor for antigen.[17,18] Control cells were incubated with Leu 1 monoclonal antibody, which recognizes a determinant (CD5) distinct from the antigen receptor. CD3-triggered and CD5-triggered cells were cultured in 15% fetal calf serum in the presence or absence of 10^2 U/mL IL-2. At time 0 and after 1, 2, 4, 5, 6, and 8 days, cell samples from the four culture conditions (CD3 or CD5 incubations ± rIL-2) were stained with OKT3-FITC and anti-IL-2 receptor 2A3 (IgG_1, Becton Dickinson, Mountain View, CA) phycoerythrin antibodies. Lymphocyte-gated cells (10^5 cells per sample) were analyzed with two-color flow cytometry on an Ortho (Raritan, NJ) fluorograph 2150.

Functional assessment of IL-2 receptor blockade by anti-IL-2 receptor monoclonal antibody. Bone marrow T cells were recovered from the bound fraction following indirect solid-phase immunoabsorption with CD5 monoclonal antibody and incubated with anti-CD3 (OKT3) monoclonal antibody (1.25×10^{-7} g/10^6 T cells) to induce surface expression of the IL-2R. Control T cells were incubated with anti-CD5 antibody. Both T cell populations, IL-2 receptor-positive and IL-2 receptor-negative, were cultured in methylcellulose in duplicate in the absence or presence of various concentrations of recombinant IL-2 (10^1 to 10^3 U/mL). Culture conditions were identical to those of the clonal erythroid progenitor assay. T cell proliferation at day 14 was assessed by enumeration of three to five fields at 10× magnification, employing an inverted microscope and a microcytometer. T cell proliferation was also assessed in the absence and presence of various concentrations of anti-IL-2 receptor monoclonal antibody.

Recombinant IL-2 caused a consistent dose-dependent proliferation of CD3-triggered T cells in methylcellulose culture. CD3-stimulated T cells increased from 43.5 ± 16.5 per three microcytometer fields in the absence of rIL-2 to 457.5 ± 2.5 in the presence of 10^3 U/mL recombinant IL-2 after 12 days in culture. In contrast, CD5-triggered adult marrow T cells were 46.5 ± 12.2 after 12 days of culture in the presence of 10^3 U/mL rIL-2. Anti-IL-2 receptor antibody (Tac-1, IgG_{2a}, provided by Dr T. Waldmann, NIH, Bethesda, MD) caused a consistent dose-dependent inhibition of recombinant IL-2 (10^2 U/mL)-induced T cell proliferation with complete abrogation at 10^{-6} g/mL antibody.

Assessment of IF-gamma release in response to rIL-2. IF-gamma was assessed by solid-phase radioimmunoassay (Centocor, Malvern, PA) in supernatants of CD5- or CD3-triggered T cell-containing marrow NAB cells (10^6/mL) cultured in the presence and absence of 10^2 U/mL rIL-2. T cell content of NAB marrow fractions was assessed by indirect immunofluorescence with pan-T Leu 4 monoclonal antibody. IF-gamma secretion in response to 10^2 U/mL rIL-2 was also assessed in the supernatants of purified CD3-triggered cultured marrow T cells (10^3 to 10^6/mL). IF-gamma concentrations were assessed following three to nine days of target cell incubation at 37°C.

Clonal progenitor assay. Bone marrow monocyte and T cell-depleted mononuclear target cells (NAB-T-enriched progenitors) were cultured alone or cocultured with CD3-triggered autologous T cells. A selected lot of Mo conditioned medium (derived from a HTLV-II-infected T lymphoblast line, kindly provided by Drs J. Gasson and D. Golde, UCLA[4]) pretested for IF-gamma and IL-2 content was employed as a source of exogenous burst-promoting activity (BPA) and was added at a final concentration of 1%. The final concentration of IF-gamma and IL-2 in culture due to the addition of Mo-conditioned medium were <0.1 U/mL. Cultures containing 0.25 U/mL purified human urinary erythropoietin (containing no detectable BPA; specific activity $>10^3$ U/mg; Terry Fox Laboratories, Vancouver, BC),[25] 15% fetal calf serum, Iscove's modified Dulbecco's medium, and 1.1% methylcellulose with and without exogenous BPA were established at a volume of 0.25 mL in cluster plates. Clonogenic progenitors giving rise to erythroid bursts (BFU-E) were enumerated at days 12 to 14 as previously described.[7] In control experiments serum concentrations from 0% to 30% and bovine albumin concentrations from 0% to 1% were studied in the absence and presence of exogenous erythroid burst-promoting activity to facilitate detection of BPA. In the presence of 15% serum and no additional albumin the addition of 1% Mo conditioned medium increased BFU-E expression from 0 to 179 ± 28 BFU-E per 1.5×10^5 NAB-T marrow cells ($p < 0.01$). Higher concentrations of Mo-conditioned medium did not provide additional BPA.

Marrow target cells were cultured at 1.5×10^5/mL as derived from experiments assessing linearity of clonogenicity over varying target cell concentrations under optimal concentrations of BPA. The ratio for NAB-T:T was 1:0.2. Adherent cells were also cocultured at an NAB-T:adherent cell ratio of 1:0.2.

Experimental design to assess lymphokine modulation of erythroid progenitor expression. Erythropoietic progenitor expression in the presence of 0 to 10^3 U/mL recombinant DNA-derived IL-2 was studied in the absence of T lymphocytes, in the presence of CD3-triggered (IL-2 receptor-positive) autologous T cells, and in the presence of control CD5-triggered (IL-2 receptor-negative) autologous T cells to assess T cell and IL-2 receptor-dependent and -independent modulation of progenitor expression by IL-2. Progenitor expression under the various concentrations of rIL-2 in each of these three coculture conditions was compared to an internal control containing the excipient control and the identical autologous T cell population. These experiments were performed in the absence and presence of exogenous BPA to assess the interaction between BPA and IL-2. With the limited serum content employed in our system, marrow BFU-E could be cloned only in the presence of exogenous Mo-derived BPA or in the presence of albumin. In control experiments T cells under identical culture conditions were shown either to consistently express (CD3-triggered cells) or not express (CD5-triggered cells) the IL-2 receptor.

Experiments with rIL-2 and IL-2 receptor–positive autologous T cells were also performed in the presence of an IL-2 receptor-blocking antibody. These experiments were designed to assess whether the effects of IL-2 on BFU-E that are observed in the presence of IL-2 receptor–positive T cells can be abrogated by blocking the IL-2 receptor on interleukin receptor–positive T cells. Results were compared with internal controls containing IL-2 receptor–positive T cells and anti–IL-2 receptor antibody but no recombinant IL-2. The results of control experiments, in which we assessed T cell proliferation under culture conditions identical to those in our clonal progenitor assay, indicated that the IL-2 receptor antibody effectively blocked rIL-2–mediated proliferation of IL-2 receptor–positive T cells and also indicated the concentration of antibody required to block the effects of rIL-2 on IL-2 receptor–positive T cells.

To assess the effects of IF-gamma on adult erythropoiesis, adult marrow progenitor expression under concentrations of recombinant DNA-derived IF-gamma ranging from 0 to 10^3 U/mL was studied in the absence of monocytes and T cells, in the presence of autologous non–CD3-stimulated T cells, in the presence of both autologous non–CD3-stimulated T cells and adherent cells, and in the presence of autologous CD3-stimulated T cells alone. Progenitor expression in the presence of IF-gamma under each of these four coculture conditions was compared to an internal control containing the identical autologous accessory cell and target cell populations plus the recombinant IF-gamma diluent. These experiments were designed to elucidate the contribution of T cells, of adherent cells (antigen-presenting cells), and the triggering of the T cell antigen receptor in modulating IF-gamma effects on erythropoiesis.

Finally, we asked whether the effects of IL-2, on erythroid progenitor growth in the presence of autologous IL-2 receptor–positive T cells, are mediated either directly or indirectly by release of IF-gamma from IL-2R–positive T cells in response to rIL-2. For this purpose, erythroid progenitor expression was studied in the presence of both recombinant interleukin-2 and autologous interleukin-2 receptor–positive T cells, and in the absence or presence of various concentrations of anti–IF-gamma monoclonal antibody. These results were compared to internal controls containing IL-2 receptor–positive T cells and anti–IF-gamma antibody but without recombinant IL-2.

Anti–IF-gamma monoclonal antibody 45/6 (IgG_1) was pretested against natural IF-gamma and the recombinant IF-gamma preparation utilized in these studies (Meloy, Springfield, VA). In suspension 6.6×10^{-8} g of this antibody was capable of neutralizing 1 unit of synthetic or natural IF-gamma as assessed by solid-phase radioimmunoassay. In semisolid medium (methylcellulose) an increase of antibody concentration of up to 1 log, relative to suspension culture, was required for neutralization. The IF-gamma antibody did not cross-react with human alpha and beta interferons.

All coculture experiments were performed with autologous mononuclear cells.

Statistics. Results are expressed as mean ± standard error of the mean unless otherwise indicated. The two-sample unpaired Student's *t* test and, when appropriate, the Student's *t* test for paired observations were used in statistical analysis.[26] All probability values for testing inhibition or stimulation were determined for a one-tailed distribution.

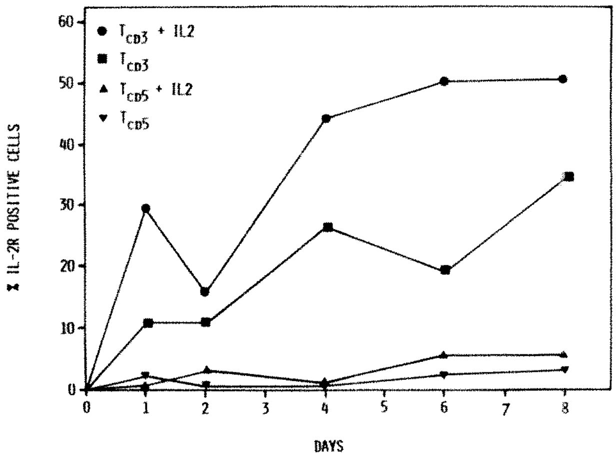

Fig 1. Induction of IL-2 receptor as assessed by cytofluorography with phycoerythrin-conjugated IL-2 receptor (IL-2R) antibody. T_{CD3}, CD3-triggered cells; T_{CD5}, CD5-triggered cells; both CD3- and CD5-triggered cells were cultured in the absence and presence of 10^2 U/mL rIL-2. Each value represents the mean of three separate experiments. 10^5 gated lymphocytes were analyzed per sample.

RESULTS

Induction of IL-2 receptor. Figure 1 depicts the kinetics of IL-2 receptor expression on adult bone marrow lymphocytes. In the presence of CD3-triggering antibody, lymphocytes continue to express IL-2 receptors for at least 1 week. Without CD3-triggering, no significant IL-2 receptor expression was observed. The addition of 10^2 U/mL rIL-2 to CD3-triggered but not to CD5-triggered T lymphocytes significantly increased both the percentage and the fluorescence intensity (data not shown) of IL-2 receptor–positive cells ($P < 0.01$). Since the intensity of fluorescence is proportional to the amount of antibody bound per cell, the increase in IL-2–receptor antibody fluorescence represents increased density of antibody binding sites per cell.

Modulation of early erythroid progenitor expression by rIL-2. Adult marrow erythroid progenitors grown in the presence of Mo conditioned medium were inhibited by rIL-2 only in the presence of autologous CD3-triggered marrow T cells (Fig 2). Compared to control cultures grown in the absence of rIL-2 but containing IL-2 receptor–positive T cells (126 ± 32 BFU-E per 1.5×10^5 NAB-T), the addition of 10 U/mL rIL-2 caused a 30% decrease, 10^2 U/mL rIL-2 caused an 86% decrease ($p < 0.01$), and 10^3 U/mL caused a 75% decrease ($p < 0.01$) in BFU-E (n = 5). rIL-2 had little stimulatory effect on adult marrow BFU-E in the presence of IL-2 receptor–negative marrow T cells (Fig 2). Adult marrow BFU-E could not be grown in the low serum assay employed in these studies in the absence of Mo conditioned medium. The addition of IL-2 receptor–negative cells plus rIL-2 did not induce BFU-E expression in the absence of Mo conditioned medium (n = 3). Serum albumin was added to cultures in subsequent experiments to overcome these growth restraints (n = 4). Under these conditions and in the absence

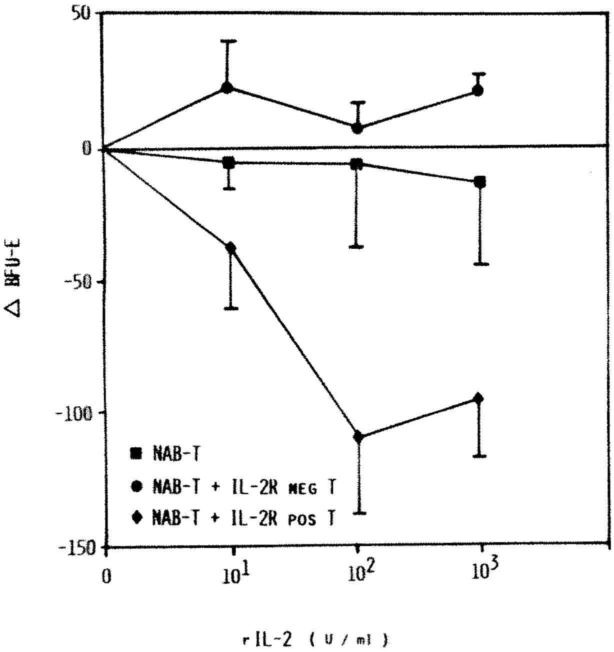

Fig 2. Effect of rIL-2 on early erythroid progenitor expression (BFU-E) fron nonadherent buoyant T-depleted (NAB-T) adult bone marrow cells cultured in the presence of Mo-conditioned medium as a source of exogenous BPA, and in the absence or presence of CD5-triggered (IL-2R–neg) and CD3-triggered (IL-2R–pos) T cells. Abscissa: final concentration of rIL-2 in culture. Ordinate: difference in absolute BFU-E numbers between rIL-2–containing cultures and control cultures. NAB-T cells were cultured at 1.5 × 10^5/mL. The ratio of NAB-T:T was 1:0.2. All BFU-E are expressed per 1.5 × 10^5 NAB-T. BFU-E controls and probability values for inhibition or stimulation were: 169 ± 63 (NAB-T, n = 5), 185 ± 65 (NAB-T plus T_{CD5}, n = 4), 126 ± 31 (NAB-T plus T_{CD3}, n = 5); $p <$ 0.01 at 10^2 and 10^3 U/mL rIL-2. Values represent mean ± SEM from four to five separate experiments.

of Mo conditioned medium, rIL in the presence of IL-2R–positive T cells (control: 185 ± 52/1.5 × 10^5 NAB-T) again caused a dose-dependent inhibition of BFU-E with 72% ± 13% inhibition ($p < 0.05$) at 10 U/mL rIL-2, 94% ± 5% inhibition ($p < 0.02$) at 10^2 U/mL, and 96% ± 4% inhibition ($p < 0.02$) at 10^3 U/mL. In the presence of IL-2R–negative cells (control: 233 ± 52) as well as in the absence of T cells (control: 207 ± 42) rIL2 again did not significantly affect BFU-E growth (80% to 95% of control at 10^2 U/mL rIL2). Thus, inhibition of BFU-E by rIL2 in the presence of IL2 receptor–positive T cells was demonstrated in both the presence and absence of Mo conditioned medium–derived BPA.

Reversal of rIL-2–mediated inhibition of erythropoiesis by anti–IL-2 receptor antibody. Inhibition of BFU-E by rIL-2 in the presence of IL-2 receptor–positive T cells was reversed by anti–IL-2 receptor antibody in a dose-dependent manner (Fig 3). Marrow erythroid progenitors, grown in the presence of 10^2 U/mL rIL-2 and autologous IL-2 receptor–positive T cells, increased from 8% ± 8% of control BFU-E in the absence of anti–IL-2 receptor antibody to 101% ± 27% of control BFU-E at 10^3 ng/mL anti–IL-2 receptor antibody (n = 3).

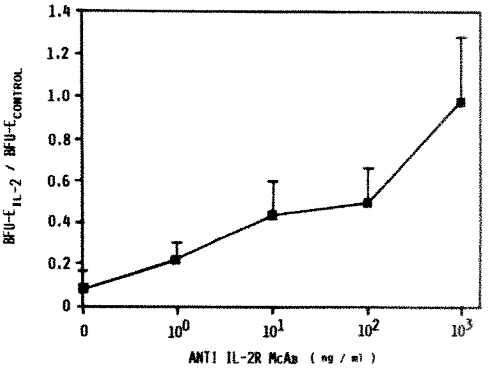

Fig 3. Effect of IL-2 receptor (IL-2R) antibody on IL-2 receptor–positive T cell–mediated inhibition of early erythroid progenitors (BFU-E) by rIL-2. Ratio of NAB-T:T was 1.0:0.2. Abscissa: final concentration of anti–IL-2 receptor antibody in culture. Ordinate: ratio of BFU-E in the presence of 10^2 U/mL rIL-2, plus IL-2R–positive T cells plus IL-2R antibody to BFU-E control in the absence of rIL2, but in the presence of IL-2R–positive T cells plus IL-2R antibody. BFU-E control per 1.5 × 10^5 NAB-T were: 124 ± 10 (adult marrow, n = 3). Values represent mean ± SEM.

rIL-2–mediated secretion of IF-gamma. We studied IF-gamma production by CD3-triggered (IL-2R–positive) and CD5-triggered (IL-2R–negative) purified marrow T cells, and by marrow NAB cells containing IL-2R–positive or IL-2R–negative T cells. IF-gamma release in the presence of 10^2 U/mL rIL-2 by 10^6/mL marrow NAB cells containing IL-2R–positive T cells ranged between 190 and 420 U/mL. In contrast, IF-gamma release by 10^6/mL marrow NAB cells containing IL-2R–negative T cells was only 1.5 to 11.5 U/mL in the presence of 10^2 U/mL rIL-2 and was 0.2 to 0.5 U/mL in the absence of rIL-2. When purified marrow IL-2R–positive T cells were assessed separately, IF-gamma production in the presence of 10^2 U/mL rIL-2 was 17 to 50 U/mL at 10^4 T cells per mL and 330 to 480 U/mL at 10^5 T cells per mL.

Inhibition of early erythroid progenitor expression by recombinant IF-gamma. Since IL-2 receptor–positive T cells release IF-gamma in response to IL-2, we studied the effects of IF-gamma on erythropoiesis and assessed its interaction with rIL-2 in our culture systems. Significant inhibition of marrow erythropoiesis by recombinant IF-gamma (rIF-gamma) required the presence of either CD3-triggered T cells alone or both CD3 nontriggered T cells plus autologous adherent cells (Table 1). Compared to control cultures grown in the absence of rIF-gamma but containing CD3-triggered T cells without monocytes (160 ± 26 BFU-E per 1.5 × 10^5 NAB-T target cells), the addition of 10^2 U/mL rIF-gamma caused a 48% decrease in BFU-E and the addition of 10^3 U/mL caused an 83% decrease ($p < 0.01$) (n = 3). In contrast, only minimal inhibition (12% to 20%) of BFU-E by 10^2 to 10^3 U/mL rIF-gamma was observed in the presence of CD5-triggered T cells alone (n = 3). However, if both CD5-triggered T cells plus autologous adherent cells were present in culture, 10^2 U/mL rIF-gamma caused a 47% decrease and 10^3 U/mL caused a 66% decrease in BFU-E ($p < 0.01$). No significant inhibition of BFU-E by rIF-gamma was demonstrable in the absence of both monocytes

Table 1. Effect of rIF-Gamma on Adult Bone Marrow Erythropoiesis

T Cells (Triggered Determinant)	Adherent Cells	rIF-gamma (U/mL)	BFU-E/1.5 × 10^5 NAB-T
—	—	0	300 ± 51
—	—	10	270 ± 48
—	—	10^2	246 ± 8
—	—	10^3	250 ± 57
T_{CD5}	—	0	294 ± 76
T_{CD5}	—	10	NT
T_{CD5}	—	10^2	214 ± 20
T_{CD5}	—	10^3	228 ± 16
—	+	0	266 ± 58
—	+	10	NT
—	+	10^2	262 ± 38
—	+	10^3	NT
T_{CD5}	+	0	272 ± 28
T_{CD5}	+	10	196 ± 0
T_{CD5}	+	10^2	136 ± 34
T_{CD5}	+	10^3	72 ± 22
T_{CD3}	—	0	185 ± 15
T_{CD3}	—	10	NT
T_{CD3}	—	10^2	70 ± 8
T_{CD3}	—	10^3	8 ± 7

Data from a representative experiment: Early erythroid progenitors (BFU-E) were scored at day 14. Mo-conditioned medium was used as a source of exogenous BPA in all experiments. Nonadherent buoyant T-depleted target cells (NAB-T) were cultured in duplicate in the absence or presence of either CD3-triggered or CD5-triggered T cells alone or in the presence of CD5-triggered T cells plus adherent cells. The ratio NAB-T:T was 1:0.2 and the final concentration of NAB-T in culture was 1.5 × 10^5 per mL. BFU-E expressed as mean SD; NT, not tested; rIF-gamma, recombinant DNA-derived interferon-gamma.

and T cells. rIF-gamma-mediated inhibition of erythropoiesis was entirely abrogated by the simultaneous or prior addition to culture of 6.6 × 10^{-5} g/mL anti-IF-gamma antibody (data not shown).

Abrogation of rIL-2-mediated inhibition of BFU-E by IF-gamma antibody. We next asked whether rIL-2-mediated inhibition of BFU-E is mediated by release of IF-gamma from autologous IL-2 receptor-positive T cells. BFU-E expression was assessed in the presence of rIL-2, IL-2 receptor-positive T cells, and anti-IF-gamma antibody (0 to 6.6 × 10^{-5} g/mL) (Fig 4). BFU-E in the presence of CD3-triggered T cells and 10^2 U/mL rIL-2 showed a dose-dependent increase from 6% ± 5% of control (180 ± 25 BFU-E) in the absence of anti-IF-gamma antibody to 52% ± 16% of control at 6.6 × 10^{-5} g/mL anti-IF-gamma antibody ($p < 0.01$) (n = 3). Control cultures in this set of experiments contained the same autologous CD3-triggered T cells plus the appropriate concentration of anti-IF-gamma antibody but no rIL-2. No substantial further augmentation in BFU-E was observed with concentrations of anti-IF-gamma antibody greater than 6.6 × 10^{-5} g/mL. BFU-E reconstituted in the presence of autologous CD3-triggered T cells plus rIL-2 and anti-IF-gamma antibody were smaller in size and less well hemoglobinized than control marrow BFU-E grown in the absence of IL-2.

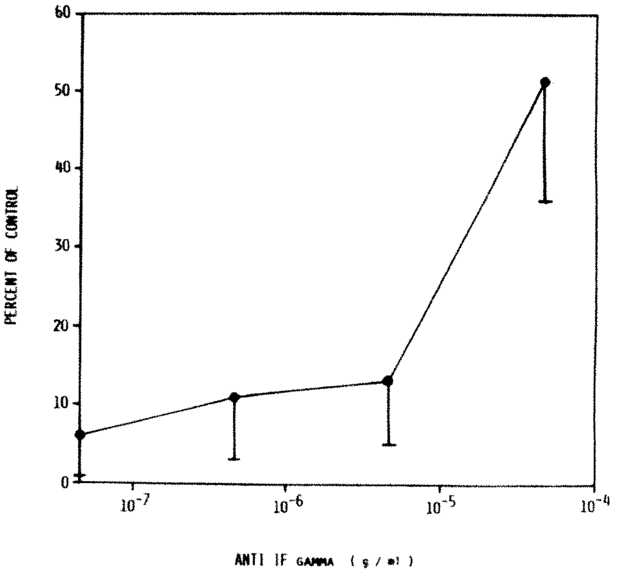

Fig 4. Abrogation of recombinant IL-2 (rIL-2)-mediated inhibition of marrow erythropoiesis by IF-gamma monoclonal antibody. Abscissa: final concentration of IF-gamma antibody in culture. Ordinate: erythroid progenitors grown in the presence of IL-2 receptor-positive T cells, 10^2 U/mL rIL-2 and various concentrations of IF-gamma antibody compared to a control cultured under identical conditions except for the absence of rIL-2. Values represent the mean ± SEM from three separate experiments. Control BFU-E: 180 ± 25 per 1.5 × 10^5 NAB-T/mL in the absence of rIL-2 and the presence of CD3-triggered T cells plus anti-IF-gamma antibody.

DISCUSSION

Our results demonstrate that (a) IL-2 receptors can be expressed on adult marrow nonadherent mononuclear cells by triggering the CD3 determinant on the T cell surface associated with the T cell receptor for antigen; (b) IL-2 does not significantly alter adult erythroid progenitor expression in the absence of T cells; (c) in the presence of CD3-triggered (IL-2-positive) autologous T cells, recombinant DNA-derived IL-2 is a potent dose-dependent inhibitor of human adult in vitro erythropoiesis; (d) blocking of the T cell IL-2 receptor abrogates the effects of IL-2 that are observed in the presence of IL-2 receptor-positive T cells; and (e) IL-2-induced inhibition of marrow erythropoiesis in the presence of IL-2 receptor-positive T cells is mediated, at least in part, by IF-gamma.

The assessment of possible differential regulatory effects of rIL-2, utilizing IL-2 receptor-positive and IL-2 receptor-negative T cells, optimally requires an assay system in which endogenous IL-2 production is blunted and in which IL-2 receptor expression can be induced independently of IL-2 production. To abrogate endogenous IL-1 release and subsequent IL-2 production, we depleted our target mononuclear cell fractions of monocytes by sequential adherence, yielding less than 1% monocyte contamination in the adherence-depleted fractions. Following monocyte depletion, endogenous IL-2 production (assessed by IL-2 bioassay) was blocked and T cell proliferation (as assessed by both proliferation in culture and by flow cytometric blast analysis of cell size and granularity [data not shown]), could be detected only following the addition of exogenous IL-2. Utilizing these assay conditions we have demonstrated an unequivocal increase in IL-2 receptor–expressing bone marrow lymphocytes by triggering a T cell antigen receptor–associated surface determinant with CD3 monoclonal antibody. These findings demonstrate that IL-2 receptor expression can occur in the absence of IL-2 production.

Previous studies utilizing fresh peripheral blood lymphocytes or peripheral blood–derived (monocyte-free) T cell clones have also suggested that IL-2 production and IL-2 receptor expression may occur independently.[19,27] In addition, sequential nuclear "runoff" experiments with lectin-activated lymphocyte populations have demonstrated that gene transcription for the IL-2 receptor precedes gene transcription for the ligand, and that IL-2 receptor mRNA can be induced independently of de novo IL-2 protein synthesis.[28] As demonstrated in our studies utilizing marrow lymphocytes and as previously demonstrated by others using adult peripheral blood mononuclear cells,[21] the addition of IL-2 increases expression of its own receptor. This secondarily induced receptor is distributed at high surface density and displays low affinity for IL-2; in contrast, the primarily induced receptor, which is expressed in the absence of IL-2, is distributed at low density on the cell surface and displays high affinity for the IL-2 ligand.[21] These observations, in toto, are most consistent with the hypothesis that expression of the primarily induced high-affinity IL-2 receptor is related to antigen presentation to T cells, whereas physiologic IL-2 production and subsequent T cell proliferation is more closely associated with the presence of (IL-1–producing) monocytes.

Inhibition of BFU-E by rIL-2 in the presence of IL-2 receptor–positive T cells was observed in the presence and absence of exogenous BPA (Mo conditioned medium). The inability of optimal amounts of exogenous BPA to overcome the inhibitory effects of rIL-2 on BFU-E in the presence of IL-2 receptor–positive T cells suggests that inhibition of BPA release is not the mechanism of rIL-2–mediated inhibition of erythroid progenitor expression. In addition, synergy or additive effects between IL-2 and other lymphokines present in Mo conditioned medium cannot account for the observed inhibitory effects of IL-2, since inhibition of erythropoiesis by IL-2 was observed in the absence of Mo conditioned medium.

As we have demonstrated, bone marrow IL-2 receptor–positive T cells release IF-gamma in response to IL-2. IF-gamma can inhibit in vitro erythropoiesis.[29,30] The role of accessory cells in mediating this effect, however, remains controversial[29,30] and the role of specific T cell antigen receptor triggering in mediating IF-gamma effects on erythropoiesis has not been previously assessed. We have demonstrated that adult marrow erythroid progenitors are inhibited by IF-gamma in the presence of both monocytes and T lymphocytes. No significant inhibition of erythroid progenitor expression was observed in the absence of both T cells and monocytes. Minimal inhibition of erythropoiesis by IF-gamma was observed with nonactivated autologous T cells in the absence of monocytes. In contrast, significant inhibition of BFU-E by IF-gamma was seen with CD3-triggered T cells in the absence of monocytes. These data suggest that triggering the T cell receptor for antigen obviates the requirement for monocytes during IF-gamma–mediated inhibition of early erythroid progenitors. Little is known about the differential expression of IF-gamma receptors on resting and antigen-triggered T lymphocytes. We hypothesize that triggering of the T cell antigen receptor, either by antigen-presenting cells such as monocytes or by anti-CD3 monoclonal antibody, renders T lymphocytes more responsive to the erythropoietic inhibitory activity of IF-gamma.

Synergy in inhibition of erythropoiesis between IF-gamma and other lymphokines (eg, IF-alpha[31] or tumor necrosis factor–beta[32]) has been demonstrated. Concomitant production of synergizing inhibitors of erythropoiesis (eg, tumor necrosis factor–beta and IF-gamma) by IL-2 receptor–positive T cells in response to IL-2 may account for the difference between the amount of rIF-gamma (10^3 U/mL) required for erythropoietic inhibition in the absence of rIL-2 and the amount of natural IF-gamma (approximately 10^2 U/mL) released by IL-2 receptor–positive T cells in response to rIL-2.

In addition to IF-gamma release by IL-2R–positive T cells in response to rIL-2, we observed that rIL-2–mediated inhibition of erythropoiesis in the presence of IL-2 receptor–positive autologous T cells can be partially abrogated by monospecific anti–IF-gamma antibody. Fifty-two percent of the erythroid inhibition caused by 10^2 U/mL rIL-2 was neutralized by adding an excess of anti–IF-gamma antibody. Our results provide evidence that release of natural IF-gamma, as a consequence of rIL-2 stimulation of IL-2R–positive T cells, plays a role in mediating rIL-2–induced inhibition of adult marrow erythropoiesis. Less than complete restoration of BFU-E colony growth by anti–IF-gamma antibody, however, with respect to both colony number and colony size, suggests that in addition to IF-gamma, either an additional lymphokine or a cellular suppressive mechanism also participates in rIL-2–induced inhibition of erythropoiesis. In addition to IF-gamma, IL-2R–positive T cells produce a variety of other lymphokines following stimulation by IL-2, eg, tumor necrosis factor–beta/lymphotoxin.[33] Tumor necrosis factor–beta has had demonstrable inhibitory activity against in vitro erythropoiesis in preliminary studies.[32]

In addition to a regulatory role in normal erythropoiesis, activated T cells[34] and T cell–derived lymphokines[35,36] have been casually implicated in various instances of bone marrow failure. IF-gamma, in particular, has been associated with the pathogenesis of aplastic anemia[35] and with the pathogenesis of red cell aplasia in the T-gamma lymphoproliferative disease syndrome.[37] A sustained pancytopenia has also been observed following IF-gamma treatment of a well-differentiated lymphocytic lymphoma.[36] Our data suggest that in addition to its effect on IF-gamma release, IL-2 in the presence of antigen-triggered T cells may directly or indirectly exert a separate inhibitory effect on erythropoiesis. IL-2 production, following lectin stimulation of in vitro T cells, was increased 100% to 900% over normal controls in 12 of 17 patients with aplastic anemia.[38] In the same study, 11 of 15 patients with aplastic anemia possessed circulating T cells bearing the IL-2 receptor, whereas circulating T cells derived from multiply transfused control patients did not express the IL-2R in the absence of exogenous lectin stimulation. The role of IL-2 in the pathogenesis of primary and secondary bone marrow failure deserves further study.

Our experiments may also bear on current clinical trials using rIL-2 in the treatment of cancer patients and patients with the acquired immunodeficiency syndrome. Infusion of rIL-2 in human subjects for 2 to 3 weeks in one study was associated with severe anemia requiring transfusion in 24 of 25 cases.[39] No evidence for accelerated red blood cell destruction was observed in this study. Treatment with rIL-2 has been associated with an increase in serum levels of IF-gamma following both continuous infusions and bolus injections of rIL-2.[40] Activated IL-2 receptor-bearing T cells, constituting up to 25% of circulating peripheral blood mononuclear cells, have also been observed in patients following infusion of rIL-2[40] and may in part mediate the observed inhibitory effects of rIL-2 on in vivo erythropoiesis. The effects of rIL-2 we have observed on in vitro erythropoiesis suggest that the anemia observed in patients treated with rIL-2 may be due, at least in part, to a specific biological effect of IL-2 on red cell production.

ACKNOWLEDGMENT

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Genetic Expression of Adenosine Deaminase in Human Lymphoid Malignancies

By T. Eng Gan, Peter E. Dadonna, and Beverly S. Mitchell

Adenosine deaminase (ADA) is an enzyme in the purine catabolic pathway that has been used as an enzymatic marker of T cell lymphoblastic malignancies due to its high specific activity in thymocytes and immature T calls. We have investigated whether the level of ADA activity in lymphoid leukemic cells correlates with the amount of ADA-specific RNA and/or immunoreactive protein in these cells as an initial step toward characterizing the nature of the genetic regulation of ADA expression during differentiation. We have found a good correlation between the steady state levels of ADA-specific RNA and ADA-immunoreactive protein in T lymphoblastic leukemic cell lines, mature T cell lines, a B lymphoblast cell line, and leukemic cells directly isolated from four patients with acute lymphoblastic leukemia and three patients with chronic lymphocytic leukemia. Southern blot analysis of DNA from these cells shows no evidence for differences in ADA gene copy number or gene rearrangement to account for the variability in ADA expression. We conclude that levels of ADA in lymphoid leukemic cells are directly related to the amount of ADA-specific mRNA present. These findings imply that ADA expression in leukemic cells reflects either the transcriptional activity of the ADA gene or the stability of ADA mRNA in these cells.

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ADENOSINE DEAMINASE (ADA; EC 3.5.4.4), a catabolic enzyme in the purine metabolic pathway, catalyzes the irreversible conversion of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively. The association of ADA deficiency and severe combined immunodeficiency disease in humans[1,2] has spurred considerable interest in the examination of this enzyme in human leukemias and lymphomas.[3-5] Although ADA is a ubiquitous mammalian enzyme, its levels vary by over 50-fold in a tissue-specific manner.[5-8] The highest levels of activity have been found in T lymphoblasts and ADA has thus been utilized as a marker of T cell malignancies.[3-5] ADA activity decreases markedly during T cell maturation,[6-7] while normal peripheral blood B cells and chronic lymphocytic leukemic cells have ADA activity that is severalfold lower than the activity in mature peripheral blood T cells.[4] It has been reported that the ADA enzyme activity found in acute lymphoblastic leukemic cells has similar biochemical and kinetic properties to ADA enzyme activity in normal peripheral blood lymphocytes,[9] implying that the alterations in ADA-specific activity in these disorders are due to alterations in the amount of ADA protein. However, the precise mechanism governing the differential expression of ADA activity in normal and malignant lymphoid cells is poorly understood. We have therefore investigated the expression of ADA at both the RNA and protein level in human lymphocytic leukemic cells to determine whether the specific activity of this enzyme directly correlates with changes in either or both of these parameters.

MATERIALS AND METHODS

Agarose, formamide, endonuclease restriction enzymes, nick-translation kits, guanidinium isothiocyanate, cesium chloride, and phenol were purchased from Bethesda Research Laboratories, Bethesda, MD. [^{32}P]dCTP, [^{125}I]Protein A and [^{14}C]adenosine were purchased from New England Nuclear, Boston, MA. All other chemicals of analytical grades were purchased from Sigma Chemical, St Louis.

Cells. Cells from three acute T cell lymphoblastic leukemia (T-ALL) cell lines (MOLT 4, MOLT 3, and RPMI 8402), two mature cutaneous T cell leukemia (T-CTCL) cell lines (Hut 78 and Hut 102), and one normal B cell line (GM 558) were studied. The cell lines MOLT-4, MOLT-3, RPMI 8402, and GM 558 were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ, and the Hut 78 and Hut 102 lines were obtained from Dr Paul Bunn, University of Colorado Medical Center, Denver. The characteristics of these cell lines have been previously reported.[10-12] In addition, leukemic cells obtained from four patients with common acute lymphoblastic leukemia antigen-positive (CALLA$^+$) acute lymphoblastic leukemia (Null-ALL) and three patients with B cell chronic lymphocytic leukemia (B-CLL) were studied, as were normal peripheral blood mononuclear cells isolated from heparinized peripheral blood by Ficoll-Hypaque gradient centrifugation.[13] Isolated mononuclear cells from patients and normal donors were tested for the following surface markers using a Coulter (Hialeah, FL) Epic V fluorescence activated cell sorter: OKT1-11 monoclonal antibodies (Ortho Diagnostics, Raritan, NJ), CALLA (J5; Orthodiagnostics), and surface immunoglobulin.

RNA determinations. Total cellular ribonucleic acid (RNA) was isolated from 1×10^8 cells using guanidinium isothiocyanate and cesium chloride gradient centrifugation.[14] Total cellular RNA was fractionated under denaturing conditions on a 2.2-mol/L formaldehyde—1% agarose gel, as previously described.[15] The fractionated RNAs were transferred to nitrocellulose filter paper according to the technique of Southern[16] and hybridized with cDNA probes nick-translated to a specific activity of 2×10^8 cpm/μg DNA. The human adenosine deaminase probes consisted of a 1.4-kb full-length cDNA sequence and a 0.65-kb Pst I fragment that contained 2/3 of the mRNA sequence for ADA from the 3'-terminal end.[17,18] The chicken β-action probe[19] was kindly provided by Dr Mark Groudine, Fred Hutchinson Cancer Center, Seattle, WA, and consisted of a 2-kb Pst I fragment containing a full-length cDNA actin sequence. After hybridization with cDNA probes, the filters were washed, dried, and exposed at −70°C to XAR-5 film for 18 to 20 hours in the presence of intensifying screens. The RNA bands specific for ADA (1.6 kb) and actin (1.8 kb) were quantitated by densitometry using a Biorad

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(Richmond, CA) scanning densitometer. Hybridization with the 0.65-kb ADA probe showed a distinct band at 1.6 kb and a variable band at 5.8 kb. Since the 1.6-kb transcript corresponds to the message for ADA in poly A–selected RNA and the 5.8-kb band appears to be nonspecific[18,20-22] we have quantitated only the 1.6-kb band for these studies. We have used the values obtained for this 1.6-kb ADA RNA band in MOLT 4 cells as a reference standard for all other cells and cell lines.

Southern blots. Genomic DNA was isolated by the method of Blin and Stafford.[23] Ten micrograms of DNA were digested with various restriction enzymes according to the manufacturer's recommendations. The digested DNA was size-fractionated on a 0.8% agarose gel and the DNA was transferred to nitrocellulose paper.[16] The filter was hybridized with both the full-length and 0.65-kb probes for ADA, washed, and autoradiographed.

Adenosine deaminase assays. The catalytic activity of ADA in dialyzed cell lysates was determined in a radiochemical assay by measuring the conversion of [^{14}C]adenosine to inosine.[24] ADA immunoreactive protein (IRP) was determined by a solid-phase radioimmunoassay. Serial dilutions of commercial calf ADA, MOLT-4 standard lysate and leukemic cell lysates were spotted in 10-μL aliquots on nitrocellulose paper (1.5 cm × 1.5 cm area) that had been previously incubated with 25 mmol/L Tris-HCl, pH 7.6, containing 0.154 mol/L NaCl (Buffer A). The filter was fixed in 10% acetic acid and 25% isopropanol for 15 minutes and rinsed several times with buffer A. The filter was then incubated with 5% BSA in buffer A overnight to block nonspecific binding of antibody, followed by a four-hour incubation at room temperature with rabbit anti-ADA serum (1:250 dilution) prepared as previously described[25] and containing 3% BSA. The filter was then washed five times for 10 minutes each in buffer A containing 0.1% Triton X-100. Subsequently, the filter was incubated with ^{125}I protein A (2×10^5 cpm/mL, specific activity 10 μCi/μg) in 50 mL of buffer A containing 3% BSA at room temperature for one hour. The filter was washed extensively with buffer A containing 0.1% Triton X-100, air-dried, and autoradiographed. The radioactive spots were cut out and counted in a Packard (Downers Grove, IL) gamma counter. Protein estimation was performed according to the method of Lowry.[26]

Western blot. Cell lysates were obtained by three cycles of rapid freeze-thawing in liquid nitrogen and the supernatants obtained after centrifugation at 10,000 × g. The supernatants were electrophoresed on 10% SDS polyacrylamide gels,[27] and the resolved proteins were transferred to nitrocellulose paper by electroelution.[28] The nitrocellulose paper was then incubated with anti-ADA antibody, probed with [^{125}I] protein A as described previously for the solid-phase radioimmunoassay, and autoradiographed.

RESULTS

The specificity of our polyclonal antibody, which was raised in rabbits, to human erythrocyte ADA for ADA in leukemic cell lysates was evaluated using a Western blot. As shown in Fig 1, a single radioactive band was visualized with MOLT-4 T lymphoblast crude cell lysate. This band corresponded to the molecular weight (M_r) of purified commercial calf ADA (Lane 5) and agrees with previously determined M_r values for the enzyme.[20,25,26] For each set of assays, a standard curve was constructed using calf ADA and MOLT-4 T lymphoblast lysate as relative internal standards. We have demonstrated on other immunoblots that this antibody cross-reacts with both the human and calf enzymes at roughly equivalent concentrations. The IRP determinations were linear from 12.5 ng to 500 ng ADA protein for the calf enzyme (Fig 2) and a similar dose–response relationship was obtained with crude lymphoblast cell lysate (data not shown).

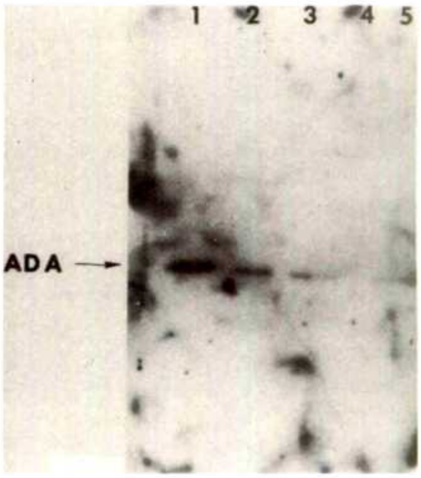

Fig 1. Autoradiographic determination of antibody specificity for ADA in MOLT-4 lysate by Western blotting. Lanes 1-3: MOLT-4 T lymphoblast lysate (lane 1, 250 μg; Lane 2, 125 μg; Lane 3, 62.5 μg). Lane 4: M_r standards phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soy bean antitrypsin inhibitor, and lactalbumin. The nitrocellulose blot containing these proteins was subsequently stained with Coomassie blue for M_r determinations. Lane 5: commercially purified calf ADA (50 ng).

A representative Northern blot used for the quantitation of ADA mRNA levels is shown in Fig 3A. The 1.6-kb band corresponds to the ADA-specific mRNA. To control for the amount of RNA loaded onto each gel, the blots were stripped and reprobed with the β-actin cDNA probe; alternatively, duplicate nitrocellulose filters were simultaneously probed with radiolabeled ADA or β-actin cDNAs (Fig 3B). The lower 1.8-kb band in Fig 3B represents actin-specific mRNA. The ADA and actin band signals were quantitated from each autoradiograph by densitometry scanning, and a ratio of ADA to actin was obtained for each sample. MOLT-

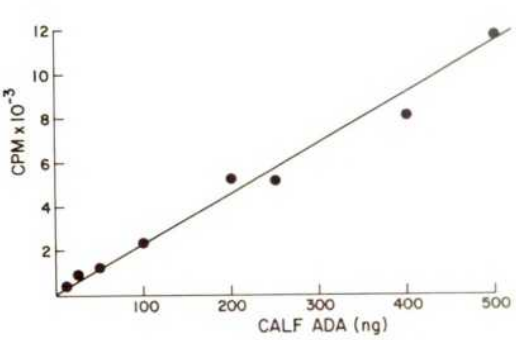

Fig 2. Standard curve for calf ADA in solid-phase radioimmunoassay. Each point represents the mean of duplicate determinations. Spots were cut from nitrocellulose filters and quantitated by gamma counting.

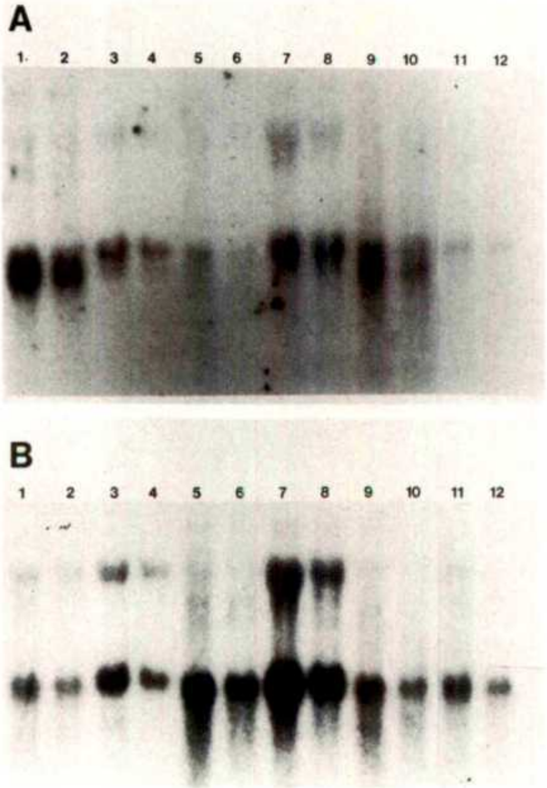

Fig 3. Quantitation of ADA-specific RNA by Northern blot analysis. (A) Hybridization of blot to 0.65-kb ADA cDNA probe. (B) Duplicate blot probed with β-actin cDNA. β-actin–specific mRNA corresponds to the 1.8-kb lower band. Lanes 1 and 2: MOLT-4 T lymphoblasts. Lanes 3 and 4: HUT 78 mature T cells. Lanes 5 and 6: HUT 102 mature cells. Lanes 7 and 8: GM 558 B lymphoblasts. Lanes 9 and 10: T cell ALL (patient). Lanes 11 and 12: normal mononuclear cells. Lanes 1, 3, 5, 7, 9, and 11 contain 10 µg RNA; Lanes 2, 4, 6, 8, 10, and 12 contain 5 µg RNA.

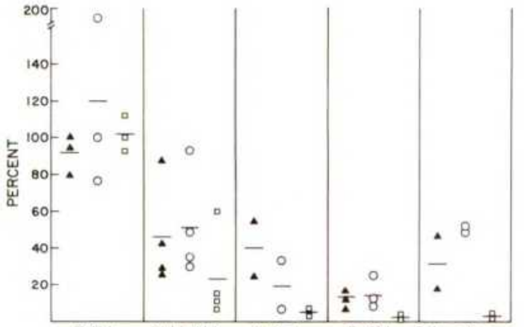

Fig 4. Histogram of relative RNA levels, IRP, and enzymatic activity for ADA in the various lymphoid malignancies. MOLT-4 T lymphoblast ADA has a specific activity of 2.06 ± 0.48 µmole/min/mg protein and an IRP value of 6.86 ± 1.62 µg/mg protein (mean ± 1 SD; six determinations). MOLT-4 ADA 1.6-kb RNA levels, IRP, and specific activity values have been used as reference standards and are expressed as 100%. ▲, mRNA; ○, IRP; □, specific activity. T-ALL: MOLT 4, MOLT 3, and RPMI 8402. Null-ALL: four patients. T-CTCL: Hut 78 and Hut 102. B-CLL: three patients. B lymphocytes: normal mononuclear cells and GM 558.

4 T lymphoblast RNA was run on each gel as an internal control and all mRNA data are expressed as a percentage of the concomitant MOLT-4 ADA:actin ratio. Figure 4 demonstrates that ADA mRNA levels and IRP values are in general good agreement for the lymphoid leukemic cells and cell lines examined. One T-ALL sample repeatedly showed a twofold increase in IRP, as shown in column one. ADA catalytic activity relative to MOLT-4 cells was equivalent in T-ALL cells, but was approximately twofold lower in other cell types. Whether this discrepancy signifies exogenous factors affecting catalytic activity or is merely attributable to the limited number of samples in each group remains to be determined.

To further investigate whether the increased levels of ADA RNA in the T lymphoblasts result from amplification or rearrangement of the ADA gene, Southern blot analyses of DNA from the various cell lines and from normal lymphocytes were performed (Fig 5). Using the full-length ADA cDNA probe, digestions of genomic DNA gave similar restriction fragments in all lanes (Figure 5, A and B), making it unlikely that the ADA gene is rearranged in these cells. Furthermore, as shown in Fig 6, an Eco RI digest on DNA from five of the cell lines reveals a single band of similar intensity in all lanes, suggesting that a variation in copy number of the ADA gene is unlikely to be responsible for the variable steady state RNA levels in the lymphoid leukemic cells studied.

DISCUSSION

The regulation of ADA expression in lymphoid cells has been of considerable interest because of the large variation in enzyme activity during lymphocyte maturation and among

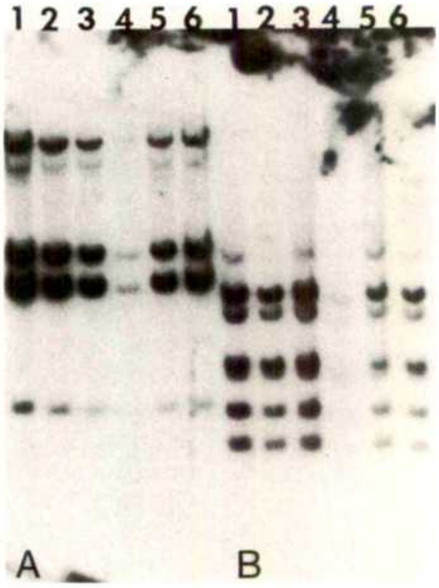

Fig 5. Southern blot analysis of genomic ADA from lymphoid cells and normal lymphocytes. Lane 1, MOLT-4 T lymphoblasts; Lane 2, RPMI 8402 T lymphoblasts; Lane 3, HUT 78 mature T cells; Lane 4, HUT 102 mature T cells; Lane 5, normal mononuclear cells; Lane 6, GM 558 B lymphoblasts. DNA (10 µg/lane) was digested with Hind III (A) and Pst I (B).

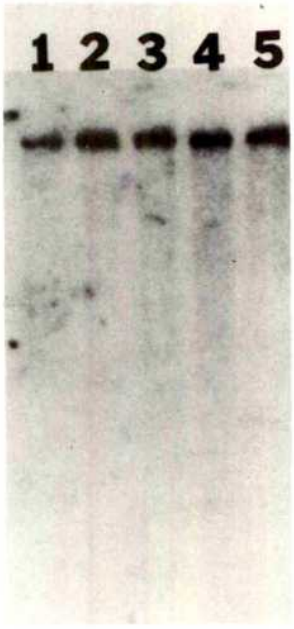

Fig 6. Southern blot analysis of genomic ADA digested with Eco RI. Lane 1, MOLT-4 T lymphoblasts; Lane 2, MOLT-3 T lymphoblasts; Lane 3, HUT 78 mature T cells; Lane 4, HUT 102 mature T cells; Lane 5, GM 558 B lymphoblasts.

lymphocyte subpopulations and because of the association of deficiency of this enzyme with immunodeficiency disease. We have investigated the genetic base of the variability in ADA expression in lymphoid leukemic cells with the ultimate objective of determining whether this variability is directly attributable to alterations at the level of gene transcription. We have found a good correlation of ADA immunoreactive protein with levels of ADA-specific RNA. These data could reflect either differences in the rates of ADA transcription or alterations in ADA mRNA stability among the leukemic cells. Differentiating between these possibilities will require direct assays of ADA transcriptional rates in isolated nuclei.[29] In either case, however, the results support the view that the high ADA activity in T lymphoblasts is determined by quantitative rather than qualitative changes in ADA protein resulting from increased levels of the corresponding mRNA. Similar correlations of enzyme protein and specific mRNA levels have been reported for dihydrofolate reductase, CAD (a multifunctional protein containing carbamyl-P-synthetase, aspartate transcarbamylase, and dihydroorotate activities) and argininosuccinate synthetase.[30-33] Our data also support the results of Adrian and Hutton,[32] who showed a close relationship among the relative ADA mRNA content, catalytic activity, and immunoreactive protein for a leukemic T and a normal B lymphoblast cell line.

Two precedents for ADA amplification have been reported, both in rodent cells that had been subjected to selective pressure for increased ADA expression. Rat hepatoma cells grown in the presence of the ADA inhibitor 2'-deoxycoformycin and the ADA substrate adenosine demonstrated increased ADA activity and mRNA levels that were due to gene amplification.[34] In addition, a mouse cell line with a 3,000-fold increase in ADA activity was demonstrated to have an equivalent increase in mRNA and gene copy number on Northern and Southern blots, respectively.[35] In this study we were unable to demonstrate any significant amplification of ADA gene sequences in human T lymphoblasts as compared to normal mononuclear cells and phenotypically more mature T_4^+ leukemic cell lines (Hut 78 and Hut 102). This is not surprising, since the high ADA activity in malignant T lymphoblasts reflects similarly high activity in normal human thymocytes. It would be difficult to reconcile the decline in ADA activity during T cell maturation with a change in the copy number of the ADA gene.

An alternative explanation for the variability in ADA expression is that the gene is rearranged during T cell development. Gene rearrangements have been documented to account for the increased transcription of the *c-myc* proto-oncogene in Burkitt's lymphoma[36,37] and are known to occur for both immunoglobulins and the T cell receptor during the respective maturation of B and T lymphocytes.[38,39] Our data, however, do not support ADA gene rearrangement in the lymphoid leukemic cells studied.

A third potential mechanism for changes in the transcription of specific genes during differentiation is alterations in the methylation status of cytosine residues in 5' regulatory regions. Hypomethylation has been correlated with an increase in corresponding mRNA levels for a variety of different proteins.[40] We have performed digestions of genomic DNA with Hpa II and Msp I restriction enzymes that show some minor differences in restriction pattern among the leukemic T cell lines when probed with the full-length ADA cDNA (data not shown). However, these changes show no consistent relationship of methylation status to ADA expression. The recent characterization of the ADA promoter[22] and sequencing of the entire ADA gene[41] should allow a more precise approach to the problems of regulation of ADA expression as a function of lymphocyte differentiation.

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Purging Leukemic Cells From Simulated Human Remission Marrow With Alkyl-Lysophospholipid

By Shinichiro Okamoto, Anita C. Olson, William R. Vogler, and Elliott F. Winton

Alkyl-lysophospholipids (ALP) are analogues of 2-lysophosphatidylcholine that have been reported to have selective antitumor activity. These compounds could potentially be useful in purging bone marrow of leukemic cells in autologous marrow transplantation in acute leukemia. To determine the efficacy of pharmacological purging by ALP, we have designed a human assay system to mimic the conditions expected in the clinical setting of autotransplantation using remission marrow. A simulated remission marrow (SRM) was prepared by mixing normal marrow cells and HL60 cells in a ratio of 1,000:1. The effect of cryopreservation on ALP-treated normal, HL60, and SRM cells was examined. In separate experiments, ALP significantly reduced the number of clonogenic HL60 cells with no effect on normal marrow progenitors. The effect of ALP was more apparent after cryopreservation. Incubation of HL60 cells with 50 μg/mL ALP for four hours followed by cryopreservation resulted approximately in a 3 log reduction of clonogenic HL60 cells. ALP also selectively purged the small number of leukemic cells from SRM. In SRM, the data suggested that ALP had indirect cytotoxic activity on leukemic cells by enhancing the cytotoxic activity of monocytes in addition to its direct effect. We found no evidence that clonogenic HL60 cells decreased because of induction of differentiation by ALP. These data indicated that treatment of marrow cells with ALP offers an efficient means to eliminate leukemic cells from the graft.

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ALLOGENEIC BONE MARROW transplantation leads to a longer disease-free survival than does chemotherapy in the patient with acute leukemia,[1,2] but in the allogeneic graft, death and morbidity often result from transplantation-related problems such as graft-*v*-host disease.[3] This procedure is rarely successful in patients more than 40 years of age[4] and is limited to patients who have HLA-matched donors.

On the other hand, autologous bone marrow transplantation (ABMT) of remission marrow has the potential advantage of being available to a larger population of patients while avoiding many of the associated immunobiological effects of allograft. The potential drawback of ABMT is contamination with microscopically undetectable leukemic cells. Thus, small numbers of leukemic cells must be purged from harvested remission marrow if the ABMT is to be curative.

Alkyl-lysophospholipid (ALP) is an analogue of naturally occurring lysophosphatidylcholine that has direct tumorcidal activity.[5,6] This activity has been reported to be selectively toxic to tumor cells including leukemic cells.[7,8] Presumably, this selectivity is due to a low or absent level of an O-alkyl cleavage enzyme capable of degrading ALP in neoplastic cells, but which is present in normal cells.[7,9,10] Without this enzyme, the compounds accumulate in the cells and inhibit phospholipid biosynthesis, which results in injury to cell membranes. In murine ABMT, treatment of murine marrow cells containing WEHI-3B leukemic cell line cells with ALP in vitro has effectively eliminated leukemic cells and spared normal stem cells, thereby allowing hematologic reconstitution.[11] Thus, these compounds may be ideal agents to purge residual leukemic cells from remission marrows.

The studies presented here were aimed at evaluating the purging effect of ALP in an in vitro human simulated remission marrow (SRM) formed by mixing normal marrow cells with human promyelocytic leukemic cells (HL60) that have been shown to be ALP sensitive[12] and by using a clonogenic assay to measure the number of residual leukemic cells and normal progenitor cells. Variables such as incubation time, concentration of ALP, and the freezing and thawing process were examined in order to establish conditions for maximal tumor cell killing without altering the viability of normal progenitor cells.

MATERIALS AND METHODS

Cells. The HL60 promyelocytic cell line[13] (obtained from Dr Robert Gallo, National Institutes of Health, Bethesda, MD) was continuously cultured (passage no., >300) in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) and passaged twice weekly. Karyotyping of the cells revealed that most metaphases contained 43 chromosomes and no cells had the Y chromosome. All studies were carried out in log-phase growth.

Normal bone marrow preparations were obtained from bone fragments provided at surgery for orthopedic procedures. The fragments were crushed and suspended in supplemented McCoy's 5A medium (GIBCO, Grand Island, NY). Single-cell suspensions were placed over Ficoll-Hypaque (Histopaque-1077, Sigma Chemical Co, St Louis) and separated by centrifugation. The interface mononuclear cells were collected and washed twice with RPMI 1640 medium and resuspended in RPMI 1640 medium with 10% FBS. In a few instances, histologically normal marrow was obtained by direct aspiration from a patient with acute leukemia in remission, and the mononuclear cells were isolated by a Ficoll-Hypaque gradient. In all instances, informed consent was obtained to donate a bone marrow specimen for research purposes.

An SRM was formed by mixing normal marrow cells obtained from male donors with HL60 cells in log-phase growth in a ratio of 1,000:1.

ALP and the treatment of cells with ALP. ALP (racemic-1-octadecyl-2-methyl-sn-glycero-3-phosphocholine, ET-18-OCH_3)

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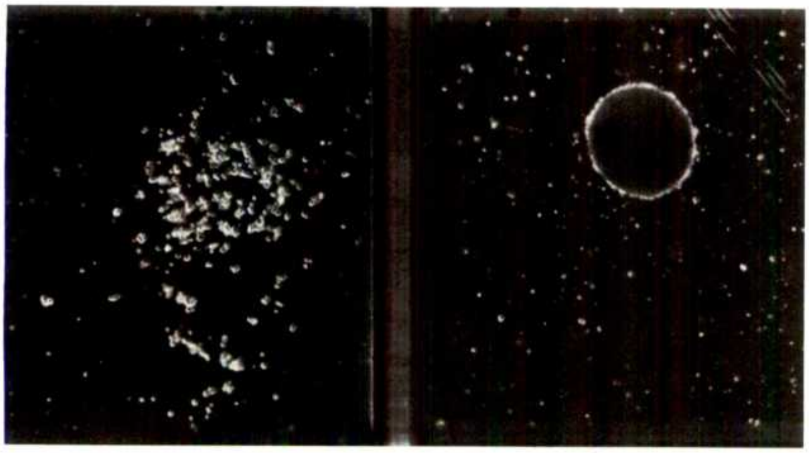

Fig 1. Colonies derived from HL60 cells (right) and GM-CFC in mixed culture of normal bone marrow cells and HL60 cells. Culture was at 37°C and 5% CO_2 for 14 days. (Original magnification × 100.)

was kindly supplied by Dr Paul Munder of the Max-Planck-Institut für Immunbiologie (Freiburg, FRG). ALP was dissolved in RPMI 1640 medium containing 10% FBS at a concentration of 1,000 μg/mL and was stored at −20°C until used.

Normal bone marrow cells, HL60 cells, and SRM cells were incubated separately at 37°C in 5% CO_2 for one and four hours in the presence of various concentrations of ALP (0, 25, 50 μg/mL). Incubation was performed in a plastic culture tube containing a 1-mL final volume at a final cell concentration of 5×10^6/mL. After the incubation period, the cells were washed twice with RPMI 1640 medium containing 10% FBS, and colony formation before and after cryopreservation was measured.

Cryopreservation and thawing of cells. The cells were suspended in RPMI 1640 medium containing 10% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma), frozen at a controlled rate of −1°C/min to −80°C, and then stored in the vapor phase of liquid nitrogen. Freezing was accomplished in freezing vials (Nunc, Roskilde, Denmark) containing a 1-mL final volume at a final cell concentration of 3 to 5×10^6/mL. Thawing of cells was accomplished rapidly by placing the vials in a 37°C water bath. After thawing, the DMSO concentration was reduced by stepwise dilution in medium to a concentration below 0.1%. Deoxyribonuclease (type I, Sigma) at a final concentration of 100 μg/mL was used when there was cell clumping.

Assay for colony formation. Mixed hematopoietic colonies (CFU-GEMM), erythroid bursts (BFU-E), erythroid colonies (CFU-E), and granulocyte-macrophage colonies and clusters (GM-CFC, GM-clusters) were assayed according to a modification of the method of Fauser and Messner.[14] The cells were cultured in medium containing 0.8% methylcellulose, 30% FBS, 0.3% bovine serum albumin, 5×10^{-5} mol/L mercaptoethanol, 5.6×10^{-8} mol/L selenium, 10% normal human serum, and the following stimulators: 10% of human placental conditioned media (HPCM),[15] 5% of PHA-stimulated lympocyte-conditioned media (PHA-LCM),[16] and 1 U/mL of erythropoietin (Step III, Connaught Laboratories, Willowdale, Ontario, Canada). One-milliliter aliquots containing 1×10^5 cells were cultured in triplicate in 35-mm culture dishes (Lux, Miles Scientific, Naperville, IL) at 37°C in a 5% humidified atmosphere. The growth of colonies on each plate was scored on day 14 of culture. Clonogenic HL60 cells were cultured in the same medium used for the assay of normal hematopoietic progenitors with or without stimulators. Before cryopreservation 1-mL aliquots containing 10^2 HL60 cells were plated, and 10^2 to 10^4 cells were plated after cryopreservation. Cell aggregates consisting of more than 40 cells were scored as colonies on day 14 of culture. SRMs were assayed for clonogenicity by the same method used for normal hematopoietic progenitors. In the SRM, HL60 cell colonies could easily be distinguished from normal GM-CFC or GM-clusters by their distinctive morphology (Fig 1). To confirm that the colonies counted were of HL60 origin, the following examinations were performed: (*a*) Each colony was aspirated, cytocentrifuged onto glass slides, and stained with Wright's Giemsa to evaluate the morphology of cells in the colonies. (*b*) Individual HL60 colonies were plucked from the plate by using a capillary pipette and placed in RPMI 1640 media without stimulators, and the regrowth of the HL60 colonies was observed. (*c*) HL60 cells were derived from a female leukemic patient; therefore, colonies derived from HL60 cells could be distinguished from male GM-CFC or GM-clusters by examining Y chromatin as per the procedure described next.

Y chromatin examination. Individual colonies were plucked and placed directly onto slides. After air drying and fixation in a 1:3 mixed acetic acid:methanol fixative, the cells were stained for 15 minutes in a 0.25% solution of quinacrine dihydrochloride (Sigma). Quinacrine solution was made by mixing a 0.5% aqueous solution of quinacrine dihydrochloride with an equal volume of citric acid–phosphate buffer (pH 5.5). The slides were rinsed in running water for one minute, coverslipped, and examined by fluorescence microscopy. Y chromatin positivity was defined as a bright fluorescent mass about 0.25 μm in diameter, peripheral and nonperipheral in location within the nucleus (Fig 2). A colony was considered Y chromatin–positive when it contained a fluorescent mass in more than 25% of the nuclei.

Preparaton of monocytes. Monocytes were isolated as described by Kumagai et al.[17] Briefly, 5 mL of the mononuclear cell suspension

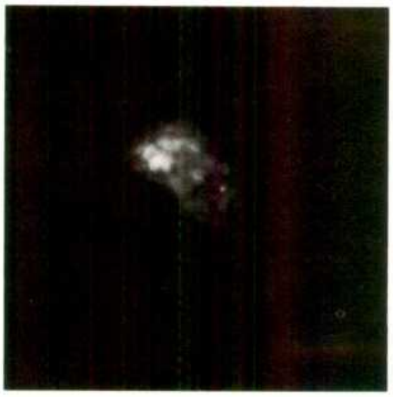

Fig 2. Granulocyte plucked from one GM-CFC and stained by quinacrine solution (see Materials and Methods). Y chromatin is seen in the nucleus. (Original magnification × 1,000.)

Table 1. Effect of ALP on Clonogenicity of HL60 Cells

ALP (μg/mL)	Colonies per 10^2 HL60 Cells	
	Without Stimulators	With Stimulators*
1-h ALP		
0	45.7 ± 4.2 (100.0) n = 19	50.7 ± 6.6 (100.0) n = 21
25	13.1 ± 2.4† (28.7) n = 18	18.0 ± 3.8† (35.5) n = 15
50	5.0 ± 0.8† (10.9) n = 15	7.2 ± 1.3† (14.2) n = 16
4-h ALP		
0	49.3 ± 7.4 (100.0) n = 11	47.2 ± 8.5 (100.0) n = 13
25	16.8 ± 4.4† (34.1) n = 11	15.7 ± 3.5† (33.3) n = 13
50	3.1 ± 1.0† (6.3) n = 11	3.5 ± 1.2† (7.4) n = 13

Results are expressed as absolute number of colonies (mean ± SE). Parentheses represent the percent survival of colonies calculated as follows: number of colonies per 10^2 ALP-treated HL60 cells × 100/number of colonies per 10^2 untreated HL60 cells.

*HL60 cells were cultured in the presence of HPCM and PHA-LCM.

†HL60 colonies significantly decreased compared with the values for untreated cells ($P < .005$).

(2×10^6 cells/mL) was pipetted into a 60-mm–diameter tissue culture dish that had been coated with 1 mL of FBS and incubated for one hour at 37°C in 5% CO_2. After incubation, the nonadherent cells were removed by aspirating the media and rinsing the dish twice with prewarmed RPMI 1640 medium. Five milliliters of phosphate buffer saline (PBS) containing 0.2% ethylenediaminetetracetic acid (Sigma) and 5% FBS were added to the dish and incubated at 4°C for 15 minutes. The adherent cells were removed by streaming PBS onto the bottom of the dish, washed twice with RPMI 1640 medium containing 10% FBS, then resuspended to the required concentration in RPMI 1640 medium containing 10% FBS. Monocytes separated by this technique had 95% viability and 97% purity by morphology.

Statistical methods. For comparison of means, the unpaired one-tailed t-test was used. A P value of $<.025$ was considered significant.

RESULTS

Effect of ALP on clonogenicity of HL60 cells. When HL60 cells were pretreated with ALP for one and four hours and cultured with PHA-LCM and HPCM or without the stimulators, clonogenic HL60 cells significantly decreased (Table 1). This effect was dose dependent, and a 94% reduction of clonogenic cells was observed when the cells were exposed to 50 μg/mL of ALP for four hours.

After freezing and thawing of HL60 cells exposed to ALP, a further reduction of clonogenic HL60 cells was noticed. As shown in Fig 3, the survival of clonogenic cells after cryopreservation decreased significantly with an increased exposure time ($P < .005$) and concentration of ALP ($P < .005$). When the cells were treated with 50 μg/mL of ALP for four hours and cryopreserved, the maximum reduction of clonogenic cells could be achieved (approximately a 99.9% reduction).

The treatment of the HL60 cells with ALP and the process of freezing and thawing did not alter the shape and cellular composition of HL60 colonies.

To determine whether the reduction of HL60 colonies could have resulted because of differentiation of HL60 induced by ALP or DMSO, the cells, after treatment with ALP or cryopreservation, were cultured in 60-mm tissue culture dishes in RPMI 1640 medium with 10% FBS for 14 days and examined morphologically on days 4, 7, 10, and 14 of culture. The short-term exposure to ALP and the process of cryopreservation did not induce significant differentiation (data not shown).

Absence of toxicity of ALP for marrow hematopoietic progenitors. Human bone marrow mononuclear cells were incubated for one and four hours with various concentrations

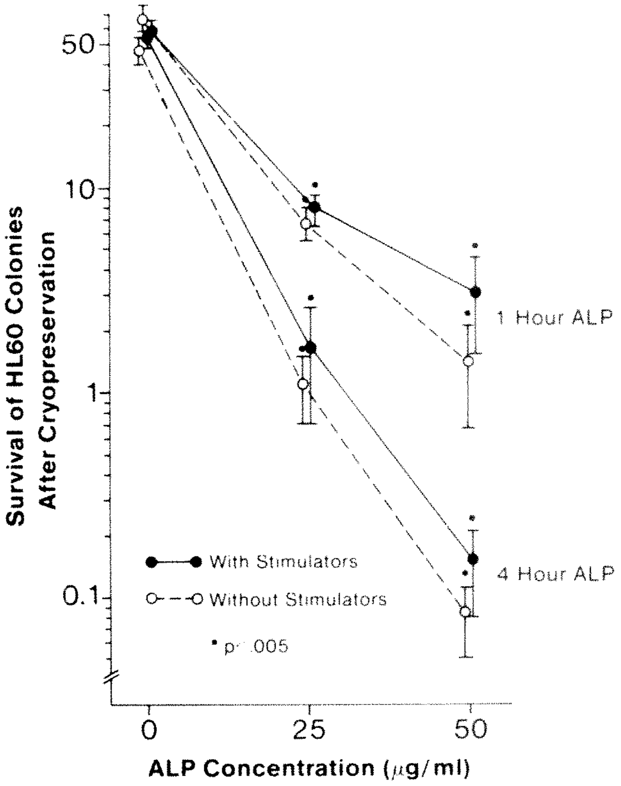

Fig 3. Percent survival of clonogenic HL60 cells after exposure to ALP and cryopreservation (see the text). After thawing, 10^2 to 10^4 treated cells were plated with (●—●) or without (○---○) stimulators. The percent survival of colonies after cryopreservation was calculated as follows: (colonies per 100 ALP-treated HL60 cells/colonies per 100 untreated HL60 cells) × (ratio of total colonies after treatment and cryopreservation to total colonies after treatment but before cryopreservation × 100). Each point represents the mean ± SE (four to six experiments were examined for culture without stimulators and six to 11 experiments for culture with stimulators).

Table 2. Effect of ALP Exposure and Cryopreservation on Survival of Marrow Progenitors

ALP (μg/mL)	Survival of Total Progenitors*	
	Before Cryopreservation	After Cryopreservation
1-h ALP		
0	100.0†	67.8 ± 5.0
25	92.0 ± 5.2	64.4 ± 12.5
50	96.5 ± 6.6	62.3 ± 9.8
4-h ALP		
0	100.0	64.6 ± 8.5
25	103.4 ± 3.8	63.1 ± 9.8
50	102.7 ± 3.5	58.5 ± 6.6

*Total progenitors refer to GM-CFC, GM-Cluster, BFU-E, CFU-E, and CFU-GEMM. The survival rate of progenitors was calculated as described in Fig 3.

†Mean ± SE for six separate marrow samples. Control marrow progenitor growth was 182.7 ± 49.3 (mean ± SE) per 10^5 marrow cells for a one-hour exposure and 143.3 ± 32.6 for a four-hour exposure.

of ALP, washed, and assayed for colony formation. As shown in Table 2, treatment of marrow cells with ALP up to a concentration of 50 μg/mL for one and four hours did not alter the total number of colonies significantly compared with untreated marrow cells.

To simulate the clinical condition of ABMT, marrow cells exposed to ALP were cryopreserved before being cultured for hematopoietic progenitors. As shown in Table 2, about 40% of the progenitors was lost through the process of freezing and thawing. However, treatment of bone marrow cells with ALP did not significantly change the survival of progenitors after cryopreservation. The morphology and composition of colonies in control and treated cultures were identical before and after cryopreservation (Table 3).

Purging effect of ALP on mixed culture of normal bone marrow cells and HL60 cells (SRM). To investigate whether ALP can kill a small amount of leukemic cells in the presence of a large number of normal bone marrow cells, experiments were performed with a mixture of 0.1% HL60 cells and 99.9% normal bone marrow cells at a concentration of 5 × 10^6/mL during treatment. For the colony assay, 10^5 normal and 10^2 HL60 cells per 1-mL plate were cultured in the presence of PHA-LCM, HPCM, and erythropoietin.

Figures 4A and 4B show that ALP significantly decreased the clonogenic HL60 cells while sparing the normal progenitors in this cell mixture both before and after cryopreservation and that no clonogenic HL60 cells were detected with the 50-μg/mL ALP concentration after four hours of treatment followed by cryopreservation. Parallel studies showed that the total number of normal colonies in mixed cell experiments was similar to those in the absence of HL60 cells (95.8 ± 4.1 for one hour and 107 ± 6.7 for four hours incubation, mean ±SE expressed as a percentage of those without HL60 cells). Also, HL60 cells did not alter the composition of normal colonies (data not shown).

As shown in Table 4, the degree of reduction of HL60 cells in SRM before cryopreservation was greater than the reduction of HL60 cells in parallel cultures of HL60 without the bone marrow cells. Also, the size of the HL60 colonies was slightly increased, and there was some peripheral halo of loosely dispersed cells around the central aggregate.

To address this observation, the influence of monocytes, one component of bone marrow cells, on the effect of ALP on clonogenic HL60 cells was examined. Monocytes obtained from peripheral blood were mixed with HL60 cells at a ratio of 10:1 and incubated for one hour in the presence of ALP. After incubation, the cells were washed twice and then measured for clonogenicity by the colony assay without stimulators.

In two experiments, the number of HL60 colonies was similar in cultures both with and without monocytes, but when the cells were treated with 12.5 μg/mL and 25 μg/mL of ALP for one hour, a greater reduction of HL60 colonies as compared with controls (Table 5) was observed in both experiments.

The presence of stimulators (PHA-LCM and HPCM) influenced neither the number of HL60 colonies (Table 1) nor the cellular composition of colonies (data not shown). Before and after cryopreservation, almost all HL60 colonies

Table 3. Effect of ALP Exposure and Cryopreservation on Composition of Progenitors

ALP (μg/mL)	Types of Progenitors				
	GM-CFC	GM-Clusters	BFU-E	CFU-E	CFU-GEMM
1-h ALP					
0 B*	27.7 ± 3.2‡	22.8 ± 3.4	40.6 ± 3.3	9.2 ± 2.3	0.5 ± 0.3
A†	24.4 ± 4.3	31.6 ± 3.5	34.5 ± 4.6	8.9 ± 2.0	0.6 ± 0.1
25 B	27.9 ± 3.9	22.9 ± 4.2	40.0 ± 5.6	8.6 ± 2.1	0.6 ± 0.2
A	24.9 ± 3.2	29.4 ± 1.7	36.2 ± 5.9	8.8 ± 1.9	0.7 ± 0.3
50 B	27.5 ± 3.8	20.6 ± 4.5	40.0 ± 4.7	11.3 ± 2.7	0.6 ± 0.2
A	26.7 ± 2.7	29.6 ± 2.6	35.2 ± 3.5	7.9 ± 1.5	0.6 ± 0.1
4-h ALP					
0 B	21.9 ± 4.9	25.3 ± 3.5	38.3 ± 5.0	13.7 ± 0.8	0.8 ± 0.3
A	21.9 ± 2.0	29.0 ± 4.1	37.0 ± 4.5	11.3 ± 2.0	0.8 ± 0.1
25 B	21.1 ± 2.8	24.8 ± 4.3	44.4 ± 6.2	9.0 ± 2.0	0.7 ± 0.3
A	22.8 ± 3.9	26.2 ± 4.1	39.3 ± 2.5	11.3 ± 1.2	0.4 ± 0.2
50 B	22.9 ± 4.4	29.5 ± 5.9	37.0 ± 6.1	10.0 ± 1.8	0.5 ± 0.2
A	20.5 ± 4.3	29.3 ± 6.6	35.6 ± 2.3	14.2 ± 1.2	0.4 ± 0.3

*Before cryopreservation.

†After cryopreservation.

‡Expressed as the percentage of the average number of total progenitors.

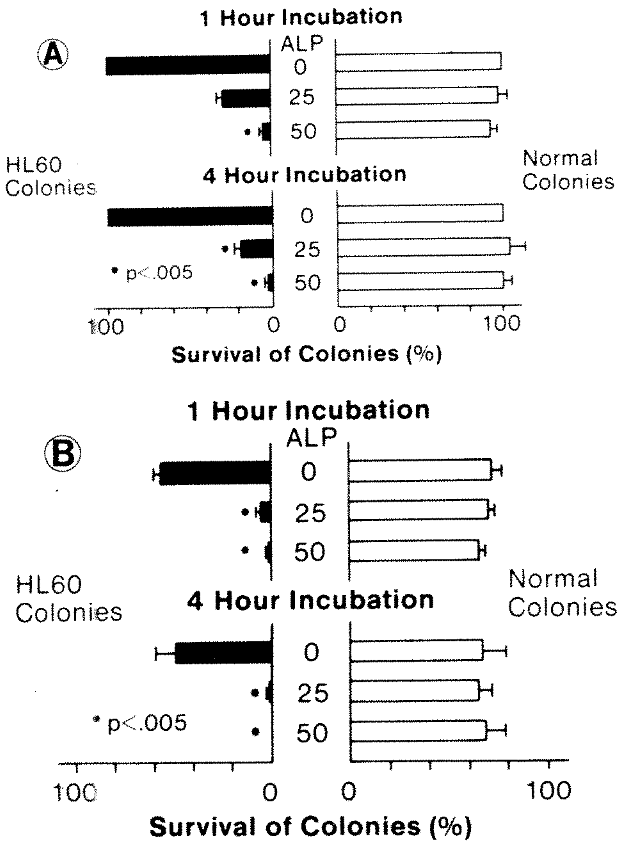

Fig 4. Effect of ALP and cryopreservation on mixed culture of normal bone marrow cells and HL60 cells. A mixture of normal bone marrow cells and HL60 cells at a ratio of 1,000:1 was cultured after treatment with ALP and (A) before or (B) after cryopreservation. Each column represents the mean percent survival of colonies (■, HL60 colonies; □, normal colonies) calculated as described in Fig 3 (±SE of the mean). Control normal progenitors' growth was 159.2 ± 44.1 (for one hour) and 143.3 ± 33.6 (for four hours) per 10^5 marrow cells; control HL60 colonies' growth was 37.4 ± 14 (for one hour) and 31.5 ± 5 (for four hours) per 10^2 HL60 cells (n = 5). Normal colonies represent the total progenitors (see Table 3).

Table 4. Effect of ALP Exposure on Clonogenicity of HL60 Cells in the Presence of Normal Bone Marrow Cells

ALP (μg/mL)	Survival (%)*
1-h ALP	
0	88.6 ± 4.9
25	70.9 ± 8.4
50	28.5 ± 4.6†
4-h ALP	
0	75.9 ± 12.1
25	30.0 ± 5.8†
50	16.1 ± 8.7†

*The percentage of survival of HL60 colonies observed in the presence of normal bone marrow cells compared with that of parallel cultures without bone marrow cells, mean ± SE for three (for four-hour ALP) and four (for one-hour ALP) separate experiments.

†$P < .005$ (comparing treated with untreated).

Table 5. Effect of ALP and Monocytes on Clonogenicity of HL60 Cells

ALP (μg/mL)	With Monocytes		Without Monocytes (Control)
	Experiment 1	Experiment 2	
1-h ALP			
0.0	66.3 ± 9.9	67.2 ± 1.5	64.3 ± 4.8
12.5	35.7 ± 0.9†	22.3 ± 0.9*	42.7 ± 1.8
25.0	10.3 ± 1.5‡	8.3 ± 1.5†	19.0 ± 2.5

Results are expressed as the absolute number of colonies per 10^2 HL60 cells and represent the mean of quadruplicate determinations for each experiment (±SE). The *P* value compares with monocytes without monocytes.

*$P < .005$.

†$P < .01$.

‡$P < .025$.

in both control and ALP-treated cultures were capable of the regrowth of HL60 cells in liquid culture. In order to ensure that HL60 cells had not been induced to differentiate to mature cells and form colonies morphologically similar to GM-CFC or clusters, the incidence of Y chromatin–positive cells in each of the colonies was investigated.

Table 6 shows the frequency of Y chromatin–positive colonies in SRM. Few Y chromatin–negative colonies were observed in granulocytic colonies. Treatment with ALP and the process of cryopreservation did not alter the frequency of Y chromatin–positive colonies. HL60 cells that were derived from a female patient were Y chromatin–negative.

DISCUSSION

No information is available stating the number of residual leukemic cells in the bone marrow after successful induction chemotherapy. Therefore, the animal or human system in which the remission marrow is simulated by using leukemic cell line cells or leukemic cells from the patients may provide a useful model for the study of ABMT in acute leukemia. Methodologies to remove residual leukemic cells from autologous marrow are being studied intensively in similar systems. These include monoclonal antibody–mediated cell lysis,[18,19] antibody-toxin conjugate,[20,21] high-gradient magnetic separation,[22] and drugs.[23,24] Some have been applied to patients clinically,[25,26] but it is still too early to evaluate the effectiveness of these procedures.

Our human model presented here has simulated the

Table 6. Y Chromatin Positivity of GM-CFC and Clusters in the SRM

ALP (μg/mL)	Y-Chromatin (+) Colonies/Colonies Examined*	
	Before Cryopreservation	After Cryopreservation
1-h ALP		
0	47/48	47/48
25	50/50	50/50
50	47/47	43/44
4-h ALP		
0	37/38	49/50
25	45/46	43/44
50	50/50	48/48

*See Materials and Methods.

remission marrow by adding HL60 cells to normal marrow cells and attempted to mimic the conditions expected in the clinical setting of ABMT in acute leukemia. Although the number of residual leukemic cells in remission marrow depends on the quality of remission, we assumed that there were up to 10^9 leukemic cells in the remission marrow with homologous distribution and that the number of cells aspirated at harvest (approximately 10^{10} cells) is about 1% of the total marrow cells.[27] From this supposition, the ratio of leukemic cells to normal cells in the graft (1:1,000) was calculated and used in this study.

Two possible drawbacks have to be considered in this system. These are the possibility of suppression of normal progenitor growth by cocultured leukemic cells and the possibility that HL60 cells can be induced to differentiate into nonclonogenic mature cells. HL60 cells have been reported to produce a leukemia-associated inhibition of progenitor growth that is biochemically indistinguishable from that of acute leukemias.[28,29] However, we did not observe an inhibitory effect on hematopoietic progenitor cell growth in our coculture. Other studies cited similar results but did find some inhibitory effects in the presence of more than 5×10^3 HL60 cells to 10^5 marrow cells.[30,31] It has been reported that the continuous exposure to a low concentration (4 μg/mL) of ALPs could induce granulocyte differentiation of HL60 cells.[32] We did not examine the continuous exposure to a low concentration of ALP. Our exposure to high concentrations of ALP for short periods revealed little differentiation-induced activity, and the process of cryopreservation using DMSO did not augment this effect.

It has been reported that colony-stimulating activity (CSA) such as PHA-LCM and HPCM could increase the number and size of HL60 cell colonies when the cells were cultured in semisolid media with CSA.[33,34] CSA also produced differentiation of cells in colonies; however, each colony still contained clonogenic HL60 cells. In our experiment, the effects of CSA were not remarkable, and it might be explained by the difference in the number of cell passages of our HL60 culture. The effect of CSA was much more obvious in earlier passages than in late passages.[34] Additionally, the presence of CSA did not alter the clonogenicity of HL60 cells treated with ALP. According to these data, one may conclude that our human system is suitable to study purging of leukemic cells from remission marrow and that the effect of ALP on HL60 cells seems to be cytoreductive rather than differentiative.

Using this system, we demonstrated that ALP had effectively purged the leukemic cells from simulated remission marrows with a sparing of normal hematopoietic progenitor cells. Unlike many chemotherapeutic agents, the cell membrane appears to be the major target of ALP. This uniqueness could explain the amplified cytotoxic effect of ALP after cryopreservation.

Ideally, the pluripotent lymphohematopoietic stem cells should be measured to assess the toxicity of the purging procedure to normal hematopoiesis. We have demonstrated indirectly that ALP could spare pluripotent stem cells in an in vivo mouse leukemic model.[11] The result of limited clinical trials of ALP did not demonstrate any hematologic toxicities and would inferentially confirm our observation.[35]

Pluripotent stem cells cannot be measured in human bone marrow at present. Most clinical and experimental investigations of ABMT used more mature progenitor assays. A correlation between GM-CFC and granulocyte recovery was observed.[36] On the other hand, full hematologic recovery was reported in patients treated with 4-hydroperoxycyclophosphamide with a dose that completely inhibited the GM-CFC growth.[26] Therefore, a more accurate assay of hematopoietic stem cells will be needed in future assays.

We observed that the cytotoxic effect of ALP was enhanced in the presence of monocytes. ALP compounds have been reported to have an antitumor activity as a result of activation of macrophages in addition to its direct cytotoxicity.[37] These studies required development of monolayers over several days, prolonged exposure to effector cells, and ALP. Some investigators observed the cytotoxic activity of monocytes after a short-term exposure of tumor cells to monocytes at a low effector-target ratio.[38,39] We did not specifically address these issues in this study. Our observation may be explained by the activation of cytotoxic monocytes by ALP or increased susceptibility of leukemic cells treated with ALP to cytotoxic monocytes. The advantage of this phenomenon in the clinical setting is an unresolved issue.

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Defect in B Cell Function in HTLV III/LAV Positive Hemophilia Patients

By E.J.M. Sjamsoedin-Visser, C.J. Heijnen, B.J.M. Zegers, and J.W. Stoop

The capacity of the peripheral blood lymphocytes (PBL) to generate an antibody response in vitro to T cell–dependent antigen ovalbumin was studied in 12 severe hemophilia patients who were otherwise in good health. PBL from four of 12 patients were not capable of generating such a response after stimulation in vitro, whereas all controls were normal. This negative plaque-forming cell (PFC) response coincided with the presence of antibodies directed toward human T-lymphotropic virus III/lymphadenopathy-associated virus (HTLV-III/LAV). Only one patient with antibodies against HTLV-III/LAV had a normal PFC response. The negative PFC response was not due to a deficient T helper cell activity, nor to an excessive T suppressor cell function. However, in the peripheral blood of these four patients, the presence of activated B cells that are refractory to antigen-specific T helper cell signals and secrete specific antibodies spontaneously could be demonstrated. Most of the patients showed a hyperimmunoglobulinemia. No correlation between the T4/T8 ratio and the level of the PFC response was demonstrable. From the data obtained in these investigations we raise the hypothesis that infection with HTLV-III/LAV in hemophilia patients will lead to in vivo (pre)activation of B cells that results in unresponsiveness or decreased response to antigen-specific signals.

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HEMOPHILIA PATIENTS are at an increased risk of developing the acquired immune deficiency syndrome (AIDS), caused by a lymphocytotropic retrovirus human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV).[1-5] Transmission occurs via biologic fluids including blood and blood products such as factor VIII and IX preparations. Aberrations of several immune functions have been demonstrated consistently in AIDS patients and notably a reversed ratio of two T cell subsets that play a pivotal role in the regulation of various types of immune responses. These subsets are characterized phenotypically by their selective affinity for the monoclonal antibodies OKT4 (T4+ cells comprising T inducer cells) and OKT8 (T8+ cells including suppressor/cytotoxic T cells).[6,7] The mechanism by which an HTLV-III/LAV infection causes the immunologic aberrations observed in AIDS is still unclear. Because of these findings in AIDS patients, hemophilia patients were studied in many countries with respect to their T4/T8 ratio. In a considerable percentage of apparently healthy patients a low T4/T8 ratio was found. In addition, they nearly always showed a hyperimmunoglobulinemia, and often the mitogenic responses of their peripheral blood lymphocytes (PBL) were low.[8-11]

In earlier studies we showed that when normal human peripheral blood T and B cells are cultured with the antigen ovalbumin (OA), small resting B cells can differentiate into plaque-forming cells (PFC) secreting small amounts of OA-specific IgM.[12] The magnitude of the PFC response is determined by the net effect of T4+ helper cell and T8+ suppressor cell activities as well as by the number of small, resting B cells present.[13-15]

On the basis of the observed in vivo alteration of immune regulatory T cell subsets and the hyperimmunoglobulinemia in many hemophilia patients, we studied the capacities of the cells (ie, T4+, T8+, and B cells) that are involved in the generation of an in vitro OA-induced PFC response.

MATERIAL AND METHODS

Patients. Twelve healthy patients with severe hemophilia A or B, ages 7 to 61 years and treated with various factor VIII or IX products, were investigated.

Factor VIII and IX concentrates of Dutch origin were used, except in patients 2 and 6. Patient 6 received concentrate from the United States (Hyland). Patient 2 who synthesized a factor VIII inhibitor received FEIBA, an activated factor IX preparation manufactured by Immuno-Vienna.

Serum immunoglobulin levels were determined by radial immunodiffusion (Mancini). Antibodies to HTLV-III/LAV were tested by using an enzyme-linked immunosorbent assay (ELISA), and ELISA-positive sera were subjected to immunoblot analysis.[16]

Controls. Included were one patient with severe hemophilia A who had not yet received any treatment at all, one patient with β-thalassaemia major treated monthly with erythrocyte concentrate, two IgG subclass–deficient patients treated with gamma globulin subcutaneously, and two hemophilia A carriers not treated with blood products. In all experiments normal control donors were included as well.

Isolation of peripheral blood mononuclear cells. The materials and methods that concern the isolation of PBL, monocytes or adherent cells (AC), T cells, and B cells have been described extensively elsewhere.[14]

Production of antigen-specific T cell–derived helper factor (ThF120-OA) and T cell–derived suppressor factor (TsF120-OA). The preparation as well as the functional and biologic properties of ThF120-OA and TsF120-OA have been described earlier.[17-19] For the preparation of ThF120-OA, isolated T cells were cultured in a Marbrook-Diener system with OA (3 μg/mL) in a concentration of 5 × 10^6 cells in 2 mL of culture medium that consisted of RPMI 1640 (GIBCO, Grand Island, NY) with 100 mmol/L glutamine, 5 × 10^{-5} mol/L 2-mercaptoethanol, antibiotics, and 2% AB serum in the presence of 10% AC for 96 hours. After this culture period the cells were washed and recultured in a concentration of 5 × 10^6 cells/mL in the presence of 3 μg OA/mL. After 24

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hours at 37°C the supernatants were harvested and tested for OA-specific T helper cell activity.

For the preparation of TsF120-OA the same protocol was applied except that 100 μg OA/mL was added to the culture during the first 96 hours and the 10% AC was omitted from the culture.[18]

Cell cultures for the generation of PFC. The culture procedures are described in detail elsewhere.[12,14] For the induction of OA-specific PFC, either PBL or purified B cells supplemented with 10% vol/vol ThF120-OA–containing supernatant were cultured for six days in the presence of 10% to 15% AC and 3 μg OA/mL. After that period the cells were harvested, washed three times, and assayed for plaque formation.

For the determination of TsF120-OA activity, 10% vol/vol TsF120-OA–containing supernatant was added to a culture of 5×10^6 PBL, 10% AC, and 3 μg OA/mL. After six days the number of PFCs was determined and compared with a control culture without TsF120-OA.

PFC assay. This method has been described in detail.[12-14] Briefly, OA-coated sheep RBC (SRBC) were centrifuged onto the bottom of flat-bottomed microtiter plates (3040, Falcon Labware, Oxnard, CA) that had been precoated with poly-L-lysine (100 μg/mL). A quantity of 0.03×10^6 viable cultured cells was added to the OA-SRBC monolayers in a volume of 20 μL and incubated for one hour in the presence of 20 μL guinea pig serum (1:20) as a complement source. The PFCs that developed were enumerated by the use of an inverted microscope.

The measurement of spontaneously antibody-producing cells. B cells prepared as described[14] were tested for the presence of PFC immediately after isolation without any culturing in vitro. B cells of patients or normal donors were transferred to a monolayer of SRBC coated with either OA or tetanus toxoid (TT) and to a monolayer of uncoated SRBC. After a one-hour incubation at 37°C, the number of PFCs was determined. The antigens OA and TT were coupled to the erythrocytes by the method of Goding.[20] Concentrations used for the coupling procedure were 1,000 μg/mL for OA and 10 lf/mL for TT.

RESULTS

Serological and immunologic characteristics in patients and controls. Table 1 summarizes the various data on the patients that are relevant to the present study. The data show normal lymphocyte counts in all patients except two who showed decreased numbers, ie, lower than 1,500/μL (patients 4 and 12). The T4+/T8+ ratio was below the control value of 0.93 (range, 0.93 to 3.43; mean, 1.79; n = 50)[21] in three patients, and most patients showed hyperimmunoglobulinemia. In five patients, specific antibodies against HTLV-III/LAV were demonstrated. In addition, all patients had serological evidence of past infections with hepatitis B virus, cytomegalovirus (CMV), and Epstein-Barr virus (EBV), respectively.

The generation of PFC in vitro. The results depicted in Fig 1 demonstrate that the PBL of four out of 12 patients could not mount a PFC response with the optimal dose (3 μg/mL) of the antigen OA: mean, 151.5 ± 96.2 PFC/10^6 lymphocytes. The eight patients with a positive PFC response showed the same level of PFCs (mean, 1,250.8 ± 281.2) as the normal control donors: mean, 1,052 ± 216 PFC/10^6 lymphocytes. The control patients also responded normally (mean, 1,199 ± 264). Since a negative PFC response may be caused by a dysfunction of antigen-presenting monocytes, T helper cells, T suppressor cells, or B cells, we designed experimental protocols in order to dissect functionally the various cellular activities. The activity of T4+ and T8+ cells was determined by their capacity to generate T helper factor and T suppressor factor respectively. T helper factor is produced by T cells after culturing the cells for 120 hours with autologous monocytes and 3 μg of the antigen OA. The supernatants of these cultures contain a factor that can only induce an OA-specific PFC response.[7,17,19] T suppressor factor is produced by T cells when they are stimulated by a high dose of the antigen (100 μg OA/mL), preferably in the absence of monocytes. Under these conditions human T suppressor cells secrete an antigen-specific T suppressor factor that is capable of decreasing the PFC response by the fact that it inhibits antigen-specific T helper cell function.[18,19] The B cell function of the patients was tested by the capacity to differentiate into PFC in the presence of OA-specific T helper factor derived from normal donor T cells.

T cell function: T helper cell activity. When B cells and 10% monocytes from a normal donor were cultured for six days in the presence of the ThF120-OA preparations of the four patients, it turned out that the ThF120-OA of each of the patients was perfectly capable of inducing the differentiation of normal B cells into PFCs (Fig 2). Because antigen presentation by monocytes is essential to generate T helper cell activity, these results prove also that the antigen-present-

Table 1. Clinical and Laboratory Features of 12 Patients With Severe Hemophilia

No.	Age (yr)	Hemophilia A/B	Therapy	IgG* (g/L)	IgM* (g/L)	IgA (g/L)	Ly (total number/μL)	T4+ (μL)	T8+ (μ/L)	T4/T8 (Ratio)	PFC Response	HTLV-III/LAV
1	7	A	FVIII conc†	13.2	1.6	1.4	2,537	1,090	608	1.79	318	+
2	8	A	FEIBA†	24.7	1.2	1.4	2,704	946	1,082	0.87	102	+
3	54	B	PCC†	26.4	3.1	1.7	1,505	421	1,159	0.36	91	+
4	61	B	PCC†	18.4	2.6	2.9	1,110	477	610	0.78	95	+
5	12	A	FVIII conc†	23.2	1.8	2.0	2,310	993	785	1.26	1,485	+
6	20	A	FVIII conc (USA)†	16.4	1.4	1.3	1,960	1,038	764	1.36	1,275	–
7	23	A	FVIII conc + cryo†	20.0	4.0	2.9	2,145	1,094	1,030	1.06	656	–
8	27	A	cryo†	12.9	2.7	2.3	1,750	892	700	1.27	1,250	–
9	28	A	cryo‡	15.0	1.5	3.1	2,162	757	800	0.95	2,045	–
10	33	B	PCC[b]	16.0	1.3	2.8	2,584	1,111	465	2.39	1,186	–
11	34	A	cryo†	12.4	1.7	3.6	2,400	912	888	1.03	1,200	–
12	48	A	cryo‡	18.2	3.4	3.1	1,127	541	406	1.02	910	–

Abbreviations: Ly, lymphocytes; conc, concentrate; cryo, cryopreservation; FVIII, factor VIII.

*Normal value for adults (mean ± 1 SD): IgG, 9.0 ± 2.17; IgM, 1.32 ± 0.46; IgA, 1.44 ± 0.55.

†Greater than 50,000 U/yr.

‡Zero to 50,000 U/yr.

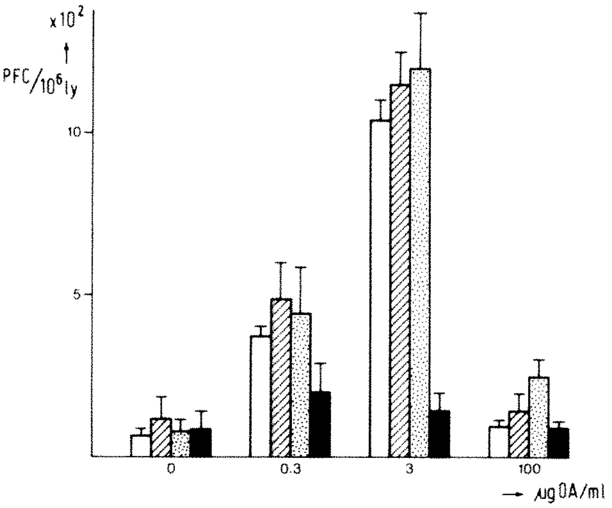

Fig 1. Antigen-dose dependency of the PFC response. A quantity of 5 × 10^6 PBL was cultured with various doses of OA. After six days at 37°C the PFC response was performed. □, normal controls (n = 52); ▨, control patients (n = 6); ▒, hemophilia patients with a positive PFC response (n = 8); ■, hemophilia patients with a negative PFC response (n = 4).

ing function of the monocytes is normal in the patients with a negative PFC response.

To demonstrate the antigen specificity of ThF120-OA, normal unprimed B cells were cultured with the various ThF120-OA preparations in the presence of the antigen SRBC. The results of the PFC response were as follows: B cells + THF120-OA of the patients + SRBC, 142 ± 7 PFC/10^6 B cells; B cells + ThF120-OA of normal donors + SRBC, 134 ± 12 PFC/10^6 B cells. These results are only slightly above the background PFC response of B cells of normal donors cultured without ThF120-OA (94 ± 11, see Table 2). We can conclude therefore that ThF120-OA produced by the patients' T cells or T cells of normal donors was specific for the antigen OA.

T suppressor cell activity. When PBL are cultured for six days in the presence of a high dose of antigen, the PFC response decreases to baseline levels (see Fig 1).[22] In previous papers we have shown that in normal donors the decline of the response is due to the activation of T suppressor-effector cells (T8+ cells).[7]

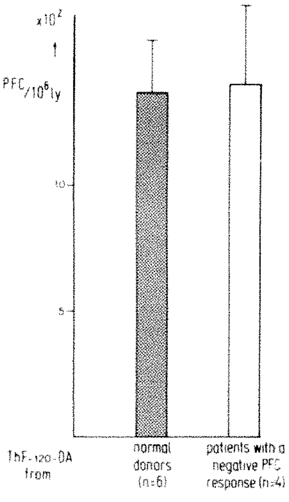

Fig 2. T helper cell activity of hemophila patients with a negative PFC response. B cells of normal donors were cultured with ThF120-OA (10%) preparations derived from the four patients with a negative PFC response (□) as well as from normal donors (▒). After six days the PFC response was performed.

Table 2. Generation of a PFC Response by B Cells of Hemophilia Patients in the Presence of ThF120-OA From Normal Donors (Mean ± SEM)

	PFC/10^6 B Cells	
B Cell Source	ThF120-OA +	ThF120-OA −
Normal donors (n = 5)	2,005 ± 39 (44 ± 3)*	94 ± 11
Patients with a negative PFC response (n = 4)	144 ± 7 (2 ± 0.4)*	45 ± 6
Patients with a positive PFC response (n = 3)	1,899 ± 21 (22 ± 4)*	64 ± 5

*The anti-SRBC PFC responses are given in parentheses. No background response is demonstrable when B cells are cultured for six days in the absence of antigen.

Because of the fact that the PFC response of four of the hemophilia patients was found to be negative, the possibility existed that the low response was due to an excessive T suppressor-effector cell function. Therefore we determined the capacity of patients' T8+ cells to produce TsF120-OA. The T suppressor cell activity was determined by adding the TsF120-OA–containing supernatant in graded quantities to normal PBL cultured with an optimal dose of the antigen OA.

The results depicted in Fig 3 show that TsF120-OA from these four hemophilia patients caused a subnormal suppression of the PFC response in low concentrations but a normal suppression in concentrations of 5% and 10% vol/vol.

B cell function: Capacity of resting B cells to differentiate into PFC. Since the monocyte, T helper and T suppressor cell functions of the patients appeared to be intact, we wondered whether the negative PFC response was due to a defect in the B cell compartment. In order to test the capacity of B cells of our patients to differentiate into PFC, we prepared ThF120-OA from T cells and monocytes of normal donors and determined the capacity of the patients' B cells to

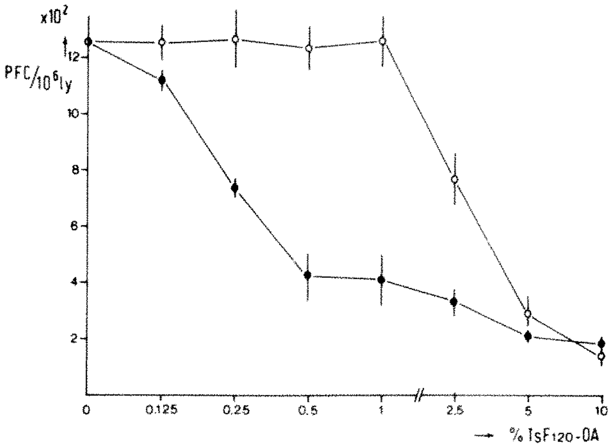

Fig 3. T suppressor-effector cell activity of hemophilia patients with a negative PFC response. PBL of normal donors were cultured with TsF120-OA preparations derived from the four patients with a negative PFC response (○) as well as from normal donors (●).

differentiate into PFC by culturing them with 10% normal ThF120-OA. The results of the PFC assay (Table 2) demonstrate that the patients whose PBL could not be induced to the formation of PFC in the presence of 3 μg OA/mL (Fig 1) did not appear to have B cells in their peripheral blood that could differentiate into PFCs in the presence of ThF120-OA.

Spontaneous IgM secretion. When normal B cells are applied directly to an OA-SRBC monolayer without any culture procedure, no specific antibody response is observed. In Table 3 it is clearly shown that, unlike the patients with a positive PFC response, the B cells present in the peripheral blood of the four hemophilia patients with a negative PFC response spontaneously secrete anti-OA antibodies. This spontaneous antibody response is not limited to an anti-OA response. We could also demonstrate the presence of B cells secreting antibodies spontaneously with specificity for sheep erythrocytes and TT (Table 3).

DISCUSSION

To obtain a better understanding of the biologic significance of the immunologic alterations found in healthy hemophilia patients, we studied the capacity of the lymphocytes of 12 patients to generate an OA-induced PFC response. The results show that in four out of 12 patients the specific antibody response in vitro against antigens like OA was negative (Table 1 and Fig 1), which could be confirmed on several occasions (data not shown). It came out that in each of these four patients an infection with HTLV-III/LAV could be shown. Only one patient with specific antibodies against this virus had a normal PFC response (Table 1).

The negative PFC response was not due to a dysfunction of the T helper cells, since every patient was capable of generating antigen-specific T helper cell factor (Fig 2). The latter finding implies that the capacity of monocytes to present antigen to T helper cells also is not affected.[17] Furthermore the proliferative response of the lymphocytes to either of the antigens TT or *Candida albicans* appeared to be positive in the patients with a negative PFC response (data not shown). We may conclude that in contrast to the data obtained in AIDS patients[23] no selective defect in the recognition of soluble antigen by T4+ cells could be demonstrated. The negative PFC response in four patients was not due to an excessive T suppressor-effector cell function either because we could show that the T cells of these patients did not produce an excess of TsF120-OA activity as compared with normal control donors, but on the contrary, they exhibited a lower TsF120-OA activity.

Table 3. Spontaneous IgM Antibody Secretion by Peripheral Blood B Cells of Hemophilia Patients Without Prior In Vitro Activation (Mean ± SEM)

	PFC/10^6 B Cells		
B Cells	αOA	αRBC	αTT
Normal donors	0	2	0
Patients with a negative PFC response (n = 4)	205 ± 69	304 ± 71	423 ± 46
Patients with a positive PFC response (n = 8)	0	5	2

Abbreviation: α, anti-.

We investigated also whether the low TsF120-OA activity was a result of a prior activation in vivo in such a way that the cells could no longer be restimulated in vitro for the production of TsF120-OA. If so, spontaneous T suppressor cells, they can be found as in the peripheral blood of agammaglobulinemic patients, for example,[24] should be present. However, no such activity could be demonstrated (data not shown) that supports our aforementioned conclusion that the negative PFC response is not caused by a direct activity of T suppressor cells.

However, we could demonstrate that the peripheral blood of patients with a negative PFC response contained B cells that spontaneously secreted antibodies of various specificities (Table 3). In each case the presence of these antibody-secreting cells in the peripheral blood coincided with an absence of a response of patients' B cells to ThF120-OA. Comparable phenomena have been described by Lane et al,[25] who reported in vivo polyclonal B cell activation and a lack of or a deficient antibody production in AIDS patients and in HTLV-III/LAV–positive patients, respectively; Pahwa et al[26] could also demonstrate a severe impairment of B cell function in these patients. More recently, increased spontaneous immunoglobulin secretion by B cells of hemophiliacs has been found in addition to decreased pokeweed mitogen–induced immunoglobulin production.[27]

't Hart et al[13] have shown recently that in our culture system only resting B cells bearing IgM and IgD on their surface are activated in vitro by OA in the presence of T helper cells or T helper cell factor. When B cells are activated (in vivo or in vitro) to the stage that they have lost their surface IgD, they are no longer sensitive to antigen-specific T helper cell signals.[28] When we consider the results presented in this paper in the light of these data, we are inclined to hypothesize that the negative PFC response is due to the fact that the B cells of these patients are no longer in a resting phase but are already activated in vivo and consequently no longer sensitive to the signals derived from OA-specific T helper cells.

Characterization of B cells secreting antibodies spontaneously as well as of the PFC that are generated in our assay system have revealed that they are not high-rate–secreting plasma cells with a blastlike appearance but are B cells that have only differentiated into the excited state (the G1 phase) of the cell cycle.[13] This transition is accompanied by a decrease in cell density and some rounds of replication during which the cell loses its surface IgD and the expression of HLA-DR increases.[13] Unanswered is the question whether these activated B cells present in HTLV-III/LAV–positive hemophilia patients play a regulatory role in vivo. Recently De Kruyff et al[29] have shown in the murine system that activated B cells play a regulatory role by the fact that they can stimulate antigen-specific T cells in the absence of antigen. The authors showed that only B cells in the G1 phase of the cell cycle possess this property. Preliminary experiments in our laboratory have already shown that B cells in an activated state can trigger T4+ cells in the absence of antigen to become T suppressor-inducer cells[30] (data not shown). Second, we have evidence that in hemophilia patients the presence of activated B cells in the peripheral blood coincides with a functional absence of T4+ suppressor-inducer cells (manuscript in preparation).

In view of the data presented here we raise the hypothesis that treatment of hemophilia patients induces activation of the immune system by transfused blood products such as factor VIII or IX concentrates and by contaminating viruses such as EBV and CMV. This stimulation of the immune system may perturb the normal homeostasis, and sometimes hyperimmunoglobulinemia and a reversed T4/T8 ratio will be the result. This state of the immune system might be susceptible to further dysregulation but need not be characterized as a pathological status per se. When, however, the treated hemophilia patient is confronted with a HTLV-III/LAV infection, this may lead to a further perturbation of the newly achieved equilibrium, which will result in more severe functional abnormalities as described here for the four patients with a negative PFC response and a positive antibody response to HTLV-III/LAV. The one patient with antibodies against HTLV-III/LAV and a normal PFC response may represent the status of a recent infection with this virus.

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Fate of the DNA in Plasmid-Containing *Escherichia coli* Minicells Ingested by Human Neutrophils

By Henry B. Fox, Pietro De Togni, Gerald McMahon, Stuart B. Levy, John S. Robinson, Morris J. Karnovsky, and Bernard M. Babior

Escherichia coli minicells containing the plasmid pSC101 ($\sim$10 kb) or pBR322 ($\sim$4 kb) were opsonized and incubated with human neutrophils. The neutrophils responded to the minicells as they would to native _E coli:_ they ingested the minicells, discharged their granule contents into the minicell-containing phagosomes, and expressed a respiratory burst. After one hour of incubation, the fate of the ingested plasmid DNA was examined. No DNA degradation was detected by trichloroacetic acid precipitation or agarose gel electrophoresis. Moreover, when pBR322 recovered from ingested minicells was transformed into _E coli,_ no mutations in either of the antibiotic resistance genes carried by the plasmid were detected out of many thousand transformants screened. These findings confirm the surprisingly limited effect of neutrophils on ingested DNA.

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THE MAJOR FUNCTION of neutrophils is to kill invading bacteria. For this purpose, the neutrophil uses a variety of microbicidal systems, some dependent on oxygen and others not. Recent work from many laboratories has elucidated the identities and modes of production of some of the microbicidal agents used by these systems, but only a few studies have dealt with the nature of the fatal lesion inflicted by these agents on the target bacteria. An elegant series of studies by Elsbach and associates has established that a potent microbicidal protein from rabbit and human neutrophils kills Gram-negative bacteria by destroying the permselectivity of their outer cell walls.[1] Investigations on oxygen-dependent killing by Thomas[2] and by Hurst and collaborators[3] have shown that the microbicidal oxidants produced by neutrophils cause the rapid oxidation of certain bacterial constituents, notably sulfhydryl groups, nucleotides, and redox enzymes bearing hemes or iron-sulfur clusters as prosthetic groups. The rate of oxidation of these constituents, however, seems to be slower than the rate of killing of the oxidant-exposed bacteria,[4] thus suggesting that the bacteria have already been fatally injured by the time those oxidation reactions have begun. Aside from these results, information on the lethal molecular lesion is rather scanty.

We have initiated studies on the molecular basis for bacterial killing by neutrophils while concentrating on the bacterial genome as a potential site of lethal damage. For these studies, we have used an experimental system that permits a detailed analysis of the action of the neutrophil on ingested DNA. This system consists of *E coli* minicells containing one to three molecules of plasmid DNA. Minicells, which are approximately one tenth the size of a normal *E coli*,[5,6] are produced during abnormal cell division of an *E coli* mutant derived by Adler et al[5] that buds from the pole of this mutant approximately every other cell division.[7] The minicells contain none of the DNA from the *E coli* genome, but they do contain DNA from plasmids in the host cell that have segregated into the minicell during its formation.[8-10] In this paper, we report on the fate of plasmid DNA from minicells that have been ingested by human neutrophils.

MATERIALS AND METHODS

Human neutrophils were prepared by dextran sedimentation and differential centrifugation as described elsewhere.[11] [^{3}H]Thymidine (50 Ci/mmol) was purchased from New England Nuclear (Boston). Media were obtained from Difco Laboratory (Detroit). Adenosine, pyridoxine, amino acids, lysozyme, pronase, RNase A, cytochrome c (type VI) and superoxide dismutase (bovine erythrocytes) were purchased from Sigma Chemical Co (St Louis). Dulbecco's phosphate-buffered saline (PBS) was purchased from GIBCO (Grand Island, NY). Agarose was obtained from BRL Laboratories, Bethesda, MD. Other reagents were the best quality commercially available and were used without further purification.

Minicells. Minicells were prepared from two plasmid-containing derivatives of the *E coli* minicell-producing strain DO-7[6]: D19-3, containing the plasmid pSC101, and D20-6, containing the plasmid pBR322. Strains were grown in L broth mixed 1:4 with ML medium[12] and supplemented with glucose (0.5%), adenosine (20 μg/mL), pyridoxine (20 μg/mL), and 10 μg/mL each of histidine, methionine, valine, and isoleucine. For labeling plasmid DNA, minicell strains were grown overnight at 37°C with aeration in [^{3}H]thymidine (4 μCi/mL). Minicells were purified from the cultures by established methods.[6,8] Residual *E coli* were destroyed by suspending the resultant minicell pellet in 200 mL of fresh L broth and incubating for 50 minutes at 37°C with penicillin (1,500 U/mL).[13] The purified minicells were then isolated by centrifugation at 12,000 g for ten minutes at room temperature, washed twice with PBS, and suspended in PBS to a concentration of 10^{10} minicells/mL ($A_{530\,nm}$ = 1.0).

Phagocytosis in minicells. Minicell uptake was measured by using minicells containing labeled plasmid DNA. Reaction mixtures contained 5 × 10^5 neutrophils and 10^9 labeled minicells in 0.2 mL PBS containing 10% (vol/vol) autologous serum. Incubations were

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carried out on a rocking platform at 37°C for the times noted. Phagocytosis was terminated with 5 mL of ice-cold 0.9% saline. Phagocytes were separated from uningested minicells by centrifugation at 50 g for ten minutes at 4°C, washed three times with ice-cold 0.9% saline, and dissolved by heating for one hour at 55°C in 0.5 mL of 0.1 mol/L NaOH. After neutralization with concentrated HCl, the solubilized neutrophils were mixed with Biofluor, and radioactivity was determined by liquid scintillation counting.

O_2^- production by neutrophils incubated with opsonized minicells. Reaction mixtures contained 1.25×10^6 neutrophils, 5×10^8 unlabeled minicells, and 0.8 mg cytochrome c in 1.0 mL PBS containing 10% (vol/vol) autologous serum. Incubations were carried out at 37°C for the times noted. Reactions were terminated with 10 μL superoxide dismutase (3 mg/mL), and O_2^- production was determined by measuring cytochrome c reduction spectrophotometrically as described elsewhere[14] against a blank containing all constituents except the neutrophils.

Cytochemistry. H_2O_2 production by neutrophils was localized by cerium staining as described by Briggs et al.[15] Neutrophils (10^7 cells) were incubated with 2×10^{10} minicells for five minutes at 37°C in 0.2 mL PBS containing 10% (vol/vol) autologous serum. Phagocytosis was stopped by adding 2 mL of ice-cold 0.1 mol/L Tris-maleate buffer (pH 7.5) containing 5% sucrose. The cells were pelleted by centrifugation at 50 g for ten minutes at 4°C and then washed twice with additional Tris-maleate. The washed cells were then incubated in a cerium-containing cytochemical medium for H_2O_2 localization[15] for 20 minutes at 37°C. Following incubation in the cytochemical medium, the cells were quickly washed twice in 0.1 mol/L Tris-maleate (pH 7.5) containing 5% sucrose and then washed once in 0.1 mol/L cacodylate buffer (pH 7.3) containing 5% sucrose. The cells were subsequently fixed for one hour at room temperature in 3% glutaraldehyde–0.1 mol/L cacodylate (pH 7.3) containing 5% sucrose. The fixed cells were washed four times in 0.1 mol/L cacodylate and then postfixed for one hour at room temperature in 2% OsO_4–0.1 mol/L cacodylate (pH 7.3). The cells were then dehydrated in a graded series of ethanols and embedded in Epon. Controls were incubated in an identical fashion except that they were not given a phagocytic stimulus.

Degranulation into minicell-containing phagocytic vesicles was evaluated cytochemically by using the myeloperoxidase stain. Neutrophils (10^7 cells) were incubated with 2×10^{10} minicells for five minutes at 37°C in PBS containing 10% (vol/vol) autologous serum. Phagocytosis was stopped by adding ice-cold 0.9% saline, and the cells were pelleted by centrifugation as described earlier. The resulting cell pellets were resuspended in 2% glutaraldehyde in PBS, fixed for 45 minutes at room temperature, and washed in PBS four times. Myeloperoxidase activity was demonstrated in the fixed cells by using the diaminobenzidine method of Graham and Karnovsky.[16] The cells were incubated in the cytochemical medium for 30 minutes at 37°C and then washed four times in 0.1 mol/L phosphate buffer (pH 7.2). Washed cells were then postfixed, dehydrated, and embedded in Epon as described before.

For electron microscopy, thin sections were cut with a diamond knife and collected on uncoated copper grids. Sections were stained with lead citrate and uranyl acetate and examined in a Philips 200 electron microscope operated at 60 kV.

Determination of stability of ingested plasmid DNA in neutrophils. Reaction mixtures containing 7.5×10^5 neutrophils and 1.5×10^9 [^{3}H]thymidine–labeled minicells in 0.3 mL PBS with 10% (vol/vol) serum were incubated for five minutes at 37°C. The reaction mixtures were then centrifuged at 50 g for ten minutes at 4°C to separate the phagocytes from the uningested minicells. The neutrophil pellet was washed once with ice-cold PBS and then resuspended in 0.3 mL PBS. The suspension was divided into two 150-μL aliquots. Half of the first aliquot was immediately spotted onto a glass fiber filter in preparation for the measurement of total radioactivity, and the other half was treated with trichloroacetic acid (TCA) (2 mL of 10% [vol/vol] acid) followed by bovine serum albumin (1.0 mL of a solution containing albumin at 10 mg/mL) and then centrifuged at 800 g for ten minutes at 4°C. The second aliquot was incubated at 37°C for an additional 55 minutes and then was treated exactly like the first. The glass fiber filters and TCA precipitates were incubated at 55°C for one hour with 0.5-mL portions of 0.1 mol/L NaOH to dissolve the proteins. After neutralization with HCl, the amounts of radioactivity in the solubilized samples were determined by liquid scintillation counting using Biofluor. Radioactivity in the supernatants was determined by counting 0.5-mL portions in Biofluor and then using this figure to calculate the total. Total TCA-precipitable and TCA-soluble radioactivity was also measured in uningested minicells; 3×10^8 minicells were used for the measurements of total radioactivity as well as for the measurements of the TCA-precipitable *v* TCA-soluble counts.

Isolation of ingested plasmid DNA. Reaction mixtures contained 2.5×10^6 neutrophils and 5×10^9 minicells (labeled with [^{3}H]thymidine for the gel electrophoresis experiments, unlabeled for the transformation experiments) in 0.5 mL PBS containing 10% (vol/vol) autologous serum. Incubations were carried out in 1-mL Eppendorf tubes for one hour at 37°C. Incubations were terminated by chilling in melting ice, and phagocytes were separated from uningested minicells by centrifugation at 50 g for ten minutes at 4°C. The cells were washed three times with 0.9% saline and then were ruptured by treatment at 0°C with 0.5 mL of lysis buffer (0.34 mol/L sucrose, 20 mmol/L Tris Cl^- [pH 7.4], 25 mmol/L KCl, 20 mmol/L Na_2 EDTA, and 0.75% [vol/vol] Triton X-100). The neutrophil nuclei, which remained intact during this treatment (as determined by DNA measurement), were removed by centrifugation at 800 g for ten minutes at 4°C. The supernatant was then mixed with a 0.1 vol of lysozyme solution (final concentration, 1 mg lysozyme/mL) and incubated for 15 minutes at 37°C to begin the dissolution of the minicells.[17] Dissolution was completed by adding 0.1 vol each of pronase and RNAse A (final concentrations, 0.2 mg/mL and 50 μg/mL, respectively) and incubating for an additional 2.5 hours at 37°C. The solution was then mixed with 0.1 vol of DNA extraction buffer (200 mmol/L NaCl and 10 mmol/L Na_2EDTA) and extracted twice with phenol/$CHCl_3$/isoamyl alcohol (24:24:1, vol/vol). DNA was then precipitated from the aqueous layer by adding 2 vol of ice-cold 95% ethanol and allowing the mixture to stand overnight at −20°C.[18] The precipitated DNA was isolated by centrifugation for ten minutes at full speed in an Eppendorf centrifuge at 0°C and then dried and dissolved in 20 μL of 10-1 storage buffer (10 mmol/L Tris chloride, pH 8.0, and 1 mmol/L Na_2EDTA). The same procedure was used to extract DNA from the control ingested minicells that had been incubated in PBS for one hour at 37°C except that the minicells were pelleted by centrifugation at 12,000 g for ten minutes at room temperature before adding lysis buffer.

Agarose gel electrophoresis. Ten-microliter portions of minicell DNA in 10-1 storage buffer were subjected to electrophoresis on 1% neutral (Tris-acetate-EDTA buffer) and alkaline agarose gels as described elsewhere.[19] The location of the DNA was determined by slicing the gel into 1-cm lengths, placing the slices in scintillation vials, adding 0.5 mL 1 mol/L HCl, heating briefly in a microwave oven to melt the agarose, and measuring the radioactivity in a liquid scintillation counter with Biofluor as the scintillant.

Detection of mutations. Mutations in the antibiotic resistance genes of pBR322 were sought by replica plating and by selection on fusaric acid. For these experiments, *E coli* HB 101 was transformed with DNA isolated from minicells carrying the plasmid pBR322. Transformation was carried out exactly as described by Cohen et al[20] using 1 to 40 μg DNA per incubation. In some experiments, the transformed bacteria were selected on L agar containing 30 μg/mL ampicillin or 5 μg/mL tetracycline, and mutant plasmids (ie, those transforming *E coli* to resistance to one of the antibiotics but not the other) were sought by replica plating of colonies onto agar containing the antibiotic on which they were not originally grown. The antibiotic sensitivity of putative mutants was confirmed by reculture of the master colonies on appropriate agar.

For other experiments, fusaric acid was used to identify mutations causing a loss of tetracycline resistance.[21,22] The transformed *E coli* were grown on agar containing 0.3% each bacto-tryptone and yeast extract, 0.1 mol/L NaCl, 0.075 mol/L NaH_2PO_4, 0.1 mol/L $ZnCl_2$, 20 μg/mL ampicillin, and 6 or 7 μg/mL fusaric acid. (These concentrations of fusaric acid were chosen because in our system they inhibited the growth of *E coli* Hb101 carrying pBR322 [ie, amp^r tet^r] but not pUC18 [amp^r tet^s]). Growth on this agar of HB 101 carrying a mutant of pRL6 that confers resistance to tetracycline at 5 μg/mL but not 25 μg/mL was partially inhibited. Antibiotic sensitivity of colonies growing on the fusaric acid plates was confirmed by replica plating onto agars containing 5 μg/mL, 25 μg/mL tetracycline, and 20 μg/mL ampicillin.

RESULTS

Interaction between neutrophils and minicells. Experiments were conducted to determine the extent to which minicells resembled normal bacteria in their interactions with neutrophils. Opsonized plasmid-containing minicells were readily taken up by neutrophils. (Fig 1). Uptake was complete by ten minutes, and fully engorged neutrophils contained ~200 minicells per phagocyte under the conditions used. Opsonized minicells also induced the neutrophils to manufacture O_2^- (Fig 2). Rates of O_2^- production were 10% to 20% of the rate seen with phorbol myristate acetate, the most effective respiratory burst activator. With the usual particulate activators (eg, zymosan[23] or native *E coli*[11]), O_2^- production by neutrophils ceases after 20 to 30 minutes; minicell-induced O_2^- production, however, proceeded at a relatively constant rate for at least 40 minutes. Minicell uptake was not affected by the plasmid carried in the minicell, but O_2^- production was somewhat lower with pSC101-containing minicells than with pBR322-containing minicells.

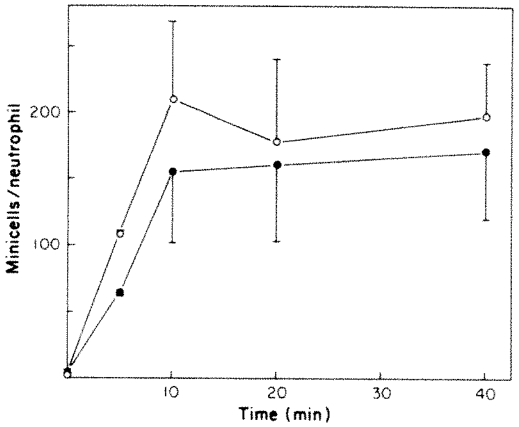

Fig 1. Uptake of minicells by neutrophils. The experiments were conducted as described in Materials and Methods. The specific activities of the minicells were 80.9 cpm/10^6 minicells for strain D19-3 (containing pSC101) (○) and 24.9 cpm/10^6 minicells for strain D20-6 (containing pBR322) (●). Results are presented as the mean ± 1 SEM of three experiments.

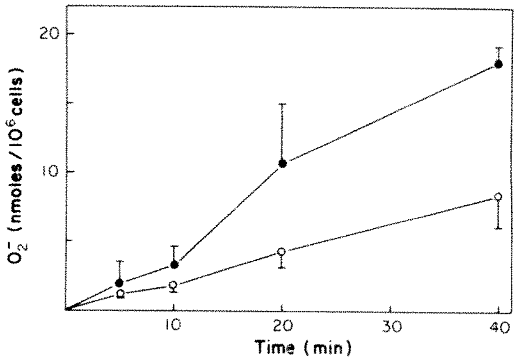

Fig 2. O_2^- production by neutrophils stimulated by opsonized minicells. The experiments were conducted as described in Materials and Methods. Results are presented as the mean ± 1 SEM of three experiments (strain D20-6) (●) or the mean ± range of two experiments (strain D19-3) (○).

Morphological studies established that most of the uptake of minicells by neutrophils represented true phagocytosis and showed further that respiratory burst oxidants were released into the minicell-containing phagocytic vesicles. This is seen in Fig 3, an electron micrograph of a neutrophil that was incubated with opsonized minicells and then treated with a stain that deposits an electron-dense precipitate at sites of H_2O_2 production. It shows that most of the neutrophil-associated minicells are contained within phagocytic vesicles, though some are attached to the cell surface. Electron-dense reaction product representing sites of H_2O_2 release was found within many of the phagocytic vesicles (~50% of the minicell-containing vesicles showed detectable reaction product) as well as over portions of the cell surface of the minicell-containing neutrophils. These results provide evidence that some of the H_2O_2 generated during the minicell-stimulated respiratory burst was actually delivered into phagocytic

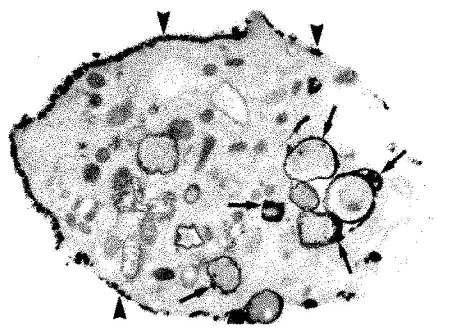

Fig 3. H_2O_2 release into minicell-containing phagocytic vacuoles. The figure shows an electron micrograph of a portion of a neutrophil that had ingested minicells for five minutes and was then stained cytochemically to localize H_2O_2 production. Note the presence of electron-dense reaction product indicating sites of H_2O_2 release within phagosomes (arrows) and on part of the cell surface (arrowheads). Uranyl acetate and lead citrate staining was used. For further details see the text.

vesicles. Reaction product was not found in control neutrophils that had not been exposed to minicells (not shown).

Granules were found to fuse with minicell-containing phagocytic vesicles, delivering their contents into the vesicle interior. This was shown by the cytochemical demonstration of the azurophil marker myeloperoxidase within the phagocytic vesicles (Fig 4). Electron-dense reaction product was seen within most phagocytic vesicles within ten minutes after initiation of particle uptake.

These findings show that an encounter between neutrophils and opsonized minicells leads to phagocytosis, degranulation, and respiratory burst activation. Neutrophils thus appear to handle *E coli* minicells in the same manner as normal bacteria.

Failure of neutrophils to degrade the plasmid within the ingested minicells. Neutrophils are equipped to degrade a variety of ingested high (protein, polysaccharide) and low (phospholipid) molecular weight substances. It therefore seemed likely that they would be able to break down the plasmid DNA molecules contained within the ingested minicells. Several experiments were performed to test this possibility.

One experiment was designed to determine whether the ingested plasmid was degraded to small fragments. In this experiment, a suspension of neutrophils that had been loaded with opsonized minicells containing the [^{3}H]thymidine–labeled plasmid pSC101 was divided into two portions. One portion was treated immediately with TCA, whereas the other portion was treated with TCA after a 55-minute incubation. The radioactivity in the precipitates and supernatants from the TCA-treated samples was then measured. The results (Table 1) show that the distribution of label between the precipitate and the supernatant was similar for the unincubated and incubated samples. Furthermore, there was much more TCA-soluble radioactivity in minicells that had never been exposed to neutrophils than there was in the ingested minicells, which suggests that most of the low–molecular weight labeled material had leaked out of the minicells shortly after they were taken up into the neutrophils, a reflection perhaps of a loss in bacterial membrane permselectivity. These results indicate that the ingested plasmid DNA was not broken down by the phagocytes to TCA-soluble fragments during the course of the incubation.

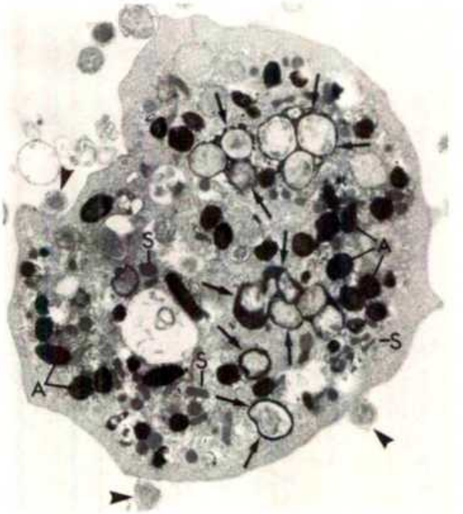

Fig 4. Discharge of myeloperoxidase into minicell-containing phagocytic vacuoles. The figure shows an electron micrograph of a portion of a neutrophil that had ingested minicells for five minutes and was then stained for myeloperoxidase. Electron-dense reaction product demonstrating myeloperoxidase activity is evident within the azurophil granules (A) and minicell-containing phagosomes (arrows). The specific granules (S) lack reaction product. Minicells bound to the surface but not yet internalized are also evident (arrowheads). Uranyl acetate and lead citrate staining was used. For further details see the text.

The foregoing experiment showed that the DNA in the phagocytosed minicells was not cut into small pieces, but it did not rule out lesser degrees of strand breakage. The possibility of limited strand breakage was investigated by taking advantage of the gel electrophoretic properties of plasmids such as pSC101.[24] In their native form, these plasmids consist of closed, circular, double-stranded DNA, extensively supercoiled. The introduction of a single nick in one of the DNA strands allows the supercoil to unwind and relax the plasmid structure. In the case of pSC101, the electrophoretic mobilities of the native and relaxed forms are sufficiently different that they are readily separated on a gel. Consequently, it is possible to detect the introduction of a single nick in the plasmid DNA by demonstrating a shift in its electrophoretic mobility from the native to the relaxed form. For the experiment, plasmid DNA recovered from neutrophils that had ingested minicells containing [^{3}H]-labeled pSC101 was purified by alcohol precipitation and subjected to agarose gel electrophoresis. Control plasmid DNA from uningested minicells was carried through the same procedure. The mobility of the plasmid DNA was determined by slicing and counting the gels. The results (Fig 5, left) show that the proportion of native to nicked pSC101 was similar in the ingested and control plasmid samples, thus indicating that few new nicks were introduced by the neutrophils into the ingested plasmid. Furthermore, little of the radioactive DNA in the ingested plasmid sample migrated more rapidly than native plasmid. This finding indicates that there was little fragmentation of the ingested plasmid into linear double-stranded pieces because these would have migrated in the gel as a broad radioactive band of high mobility. From these results, we conclude that the microbicidal systems of the neutrophil caused very little strand breakage in the ingested DNA.

A form of damage that would have been overlooked by the foregoing techniques is depurination (loss of bases without damage to the DNA sugars or backbone). Depurination alone does not cause strand breakage, so its effect on the electrophoretic mobility of the plasmid would be small. The elimination of bases, however, generates sites at which the DNA molecules will break when exposed to high pH values.[25] Accordingly, depurinated DNA subjected to alkaline electrophoresis will cleave into fragments. Because of their size, these fragments will migrate more rapidly than undamaged DNA so that the occurrence of depurination in a DNA sample may be tentatively established from the electrophoretic mobility of the DNA in an alkaline gel.

Table 1. Lack of Degradation of Ingested Plasmid DNA to TCA-Soluble Material

Duration of Incubation	Radioactivity (cpm) Precipitant	Radioactivity (cpm) In Supernate	Proportion of Radioactivity in Supernate (%)	Total Radioactivity (cpm)	Recovery* (%)
5 min	348 ± 78	327 ± 98	48.3 ± 11.4	648 ± 94	100.7 ± 4.5
60 min	286 ± 29	387 ± 87	56.6 ± 2.9	632 ± 40	105.0 ± 10.9
None	541 ± 29	2,313 ± 168	81.0 ± 0.4	2,949 ± 198	96.8 ± 1.2

Experiments were conducted as described in Materials and Methods. Results are presented as the mean ± 1 SE of three experiments.

*(cpm in precipitate + cpm in supernate)/cpm on filter.

To determine whether DNA in the ingested minicells had undergone depurination, [^{3}H]-labeled pSC101 recovered from the ingested minicells was subjected to alkaline gel electrophoresis along with a control sample of intact plasmid DNA. The results (Fig 5, right) showed that the mobility of the ingested plasmid DNA was the same as that of the control. These results strongly suggest that the ingested DNA underwent little if any depurination.

Mutation of the plasmid within the ingested minicells. The foregoing results indicate that there is little gross damage or disruption in the integrity of a plasmid DNA molecule within a minicell that has been ingested by a neutrophil. Prior studies, however, have suggested that neutrophils are able to alter DNA structure[26-29] because exposure to these cells leads to the reversion of mutant bacteria to the wild phenotype (Ames test strains of *Salmonella typhimurium* lose their histidine requirement, and light production is restored to dark mutants of the chemiluminescent microorganism *Photobacterium fischeri*) and to the appearance of sister chromatid exchanges in cultured fibroblasts. Experiments were therefore carried out to determine whether neutrophils were able to induce mutations in the DNA within an ingested minicell.

The plasmid used in these experiments was pBR322, a small plasmid (4363 base pairs [bp]) that specifies resistance to ampicillin and tetracycline.[30] Minicells containing this plasmid were fed to neutrophils, and after one hour the ingested plasmid DNA was isolated and used to transform a competent strain of antibiotic-sensitive *E coli* by selecting for antibiotic resistance by growth on plates containing either ampicillin (30 μg/mL) or tetracycline (5 μg/mL). Colonies appearing on these plates were then tested for resistance to the other antibiotic by replica plating. Since colonies derived from bacteria transformed by native pBR322 should be resistant to both ampicillin and tetracycline, any colony that was resistant to one antibiotic but sensitive to the other would

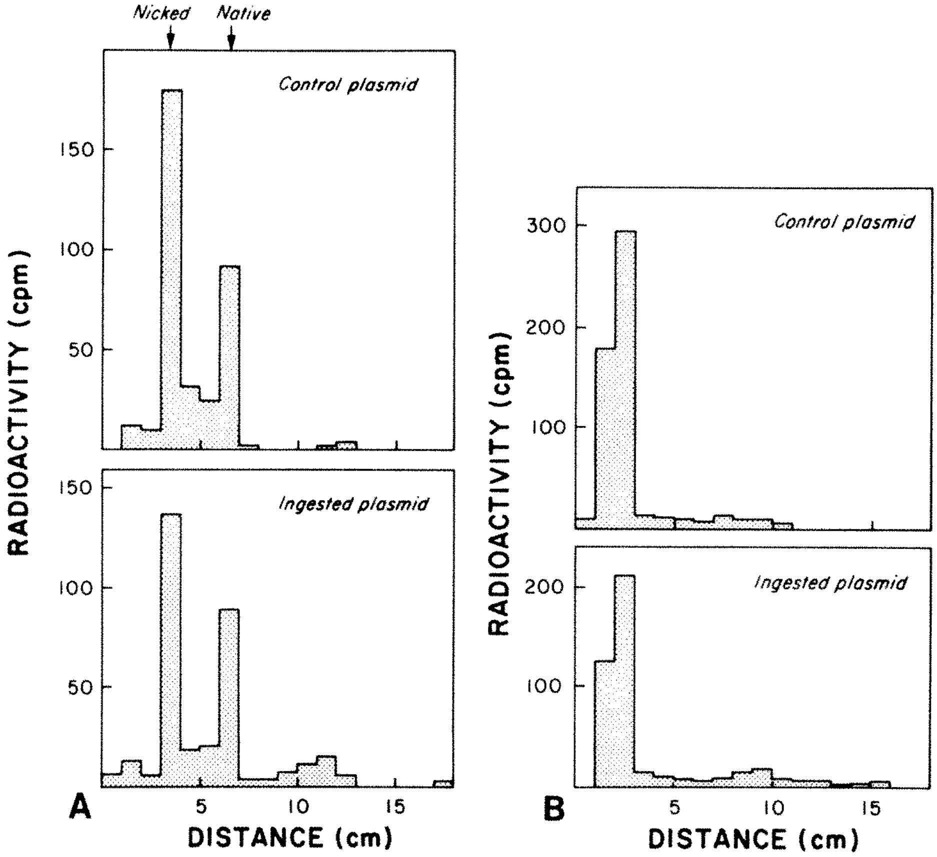

Fig 5. Gel electrophoresis of plasmid DNA from ingested and uningested minicells. Experiments were conducted as described in Materials and Methods. (A), neutral gel; (B) alkaline gel.

have to have been derived from a bacterium that had been transformed by a plasmid carrying a mutation in the gene coding for resistance to the second antibiotic.

In a representative experiment in which 1,266 transformants (663 initially selected with ampicillin and 603 with tetracycline) were obtained with control pBR322 and 1,160 transformants (589 ampicillin and 571 tetracycline) with pBR322 isolated from ingested minicells, replica plating revealed no mutants in either group. Mutants also failed to appear in three other experiments in which transformants, though not counted, were plated at a density estimated to be similar to that used in the first experiment.

Tet^s mutants were also sought by a positive selection method that allows 10^5 colony-forming units to be screened for tetracycline sensitivity with relative ease. This method is based on the ability of tetracycline-sensitive but not tetracycline-resistant *E coli* to grow on fusaric acid.[21,22] From three separate experiments, 436,000 ampicillin-resistant transformants were obtained, 96 of which were found to grow on fusaric acid. Replica plating, however, showed that none of these 96 were sensitive to tetracycline.

These results suggest that the incidence of mutation in DNA ingested by neutrophils must be quite low, although estimating an exact mutation rate is difficult because of the possible occurrence of mutations that are not detected by the technique used. In any case, these findings together with the observations described in earlier sections indicate that neutrophils have little effect on ingested DNA.

DISCUSSION

Earlier studies on the fate of DNA ingested by neutrophils have yielded results similar to those reported here. Both Elsbach's group[31] and Rozenberg-Arska et al[32] found that neutrophils had little ability to degrade *E coli* DNA, whether chromosomal (measured as the release of [^{3}H]thymidine into TCA-soluble material) or plasmid (measured as a loss of the ability to transform fresh bacteria to ampicillin resistance). Similarly, Lamers et al studying the interaction of DNA–anti-DNA complexes with phagocytes found little degradation of ingested DNA by neutrophils.[33] Our own work, which used more sensitive assays than did the earlier studies, confirms the surprisingly limited effect of neutrophils on ingested DNA. On the other hand, Birnboim and colleagues have provided evidence that endogenous DNA of neutrophils was extensively cleaved by the oxidizing agents generated by the cells when they were activated.[34-36] The apparent discrepancy between Birnboim's results and those reported here might be explained by differences in target size, which is over 100,000 times greater for the human genome (2.7×10^6 kb) than for the plasmid used in our experiments.

If it is assumed that the present findings obtained with *E coli* minicells containing plasmid DNA can be extended to the full genome of a complete *E coli*, it is then possible to use them to estimate the rate of cleavage of the genomic DNA of a microorganism that has been ingested by a neutrophil. Interpreted conservatively, the results in Fig 5 indicate that after one hour, fewer than 10% of the native plasmid DNA molecules that were associated with the neutrophils had sustained a nick. Since the plasmid used in those experiments is 10 kb in size, the neutrophil-associated plasmid DNA acquired less than 1 nick/h/100 kb. At this rate, approximately one minute would be required for half of a population of ingested *E coli* to sustain at least one nick in their 4,000-kb genomes. This level of DNA damage would ordinarily be insufficient to kill the ingested bacteria because of the existence of highly efficient mechanisms that repair such damage almost as soon as it occurs.[37] The rapid loss of low–molecular weight material from the ingested microorganisms (Table 1), however, suggests that DNA repair in these bacteria might fail owing to nucleotide deficiency. Under these circumstances, even relatively minor levels of DNA damage could contribute to the death of the ingested microorganism.

At first glance, these results would seem to be in conflict with prior work showing that activated neutrophils can induce mutations in biologic systems. There are at least two possible explanations for this apparent discrepancy. First, neutrophil-induced mutations may be of a frequency that could be detected in a $\sim 10^3$-kb (ie, bacterial) or $\sim 10^6$ (mammalian) genome but not in the tiny pseudogenome represented by a plasmid. Alternatively, it may be that mutations occur only when extracellular targets are exposed to activated neutrophils and are not seen in intracellular (ie, ingested) neutrophil targets. In earlier studies of neutrophil-induced mutagenesis, extracellular targets have been uniformly used.[26-29]

The resistance of plasmid DNA to destruction by neutrophils raises the question as to the ultimate fate of plasmids carried by bacteria that are ingested by these cells. Some of these plasmids may be taken up and destroyed by mononuclear phagocytes, which have been shown in other studies to be capable of degrading DNA by nuclease action.[32,33] It seems likely, however, that at least a few will be released intact into the tissues. What then happens to these liberated units of DNA remains to be determined.

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Heterogeneity of Platelet Secretion in Response to Thrombin Demonstrated by Fluorescence Flow Cytometry

By Geoffrey I. Johnston, Elaine B. Pickett, Rodger P. McEver, and James N. George

Platelet membrane changes that accompany in vivo activation may be difficult to detect if only a small fraction of circulating platelets has undergone secretion. This study describes an approach to that problem by using a method to measure the number of molecules of fluorescein-labeled antibody bound to individual platelets by flow cytometry. The platelet response to different concentrations of thrombin was determined by measuring the binding of a monoclonal antibody (S12) to GMP-140, an α-granule membrane protein that becomes exposed on the platelet surface during α-granule secretion. Unstimulated platelets bound a mean of 1,120 molecules of S12 per cell, and 93% of platelets bound less than 2,000 molecules. Platelet stimulation by 0.25 U/mL thrombin caused maximum S12 binding with a mean of 7,529 molecules per cell. Even at low concentrations of thrombin (0.025 U/mL), 5% of platelets were maximally activated, binding over 7,000 molecules of S12 per cell. Conversely, at 0.25 U/mL thrombin, 13% of platelets continued to bind less than 2,000 molecules of S12 per cell. A mixture of as little as 5% thrombin-activated platelets with unstimulated platelets could be detected by this method. Therefore flow cytometry offers an important tool for investigating patients who may have circulating activated platelets as part of a disorder predisposing to thrombosis or hemorrhage.

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PLATELET SURFACE GLYCOPROTEINS are critical for platelet–vessel wall interactions and for the formation of hemostatic platelet aggregates.[1] Activation of platelets by thrombin during these interactions causes a change in the concentration and organization of surface glycoproteins, including the appearance of an α-granule membrane protein (GMP-140) on the platelet surface.[2-7] GMP-140 is a good marker for platelet secretion because its surface expression correlates with the release of α-granule contents[5,6] and it is not reinternalized following secretion.[6]

Previous studies to identify patients with circulating activated platelets[6] measured the binding of radiolabeled monoclonal antibodies to platelets, a method that can determine only the average number of surface glycoprotein molecules per platelet in the total platelet population. Analysis of membrane changes on individual platelets would improve the ability to detect acquired platelet disorders, since only a fraction of platelets may become activated and remain in the circulation. The use of fluorescein-labeled monoclonal antibodies and flow cytometry has been an important advance in the study of surface glycoproteins on single platelets.[8-12] In this study we have further developed the flow cytometry technique by using a direct antibody binding assay to measure the number of glycoprotein molecules exposed on the surface of individual platelets.

METHODS

Platelet suspensions. Blood was collected from healthy volunteers in acid-citrate-dextrose containing prostaglandin E_1, and platelets were washed and resuspended in Tyrode's buffer, pH 7.4, containing 0.35% bovine serum albumin (BSA), 50 ng/mL PGE_1, and 2 mmol/L EDTA as previously described.[6] Samples were obtained in accordance with the guidelines of the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

Antibody preparation. Two monoclonal antibodies, Tab and S12, were used in these studies. Tab, which binds to glycoprotein IIb,[13] was dual labeled with ^{125}I and fluorescein isothiocyanate (FITC) and was used to calibrate the flow cytometer. S12, which binds to GMP-140,[2] was labeled with FITC and was used to assess platelet activation. To label monoclonal antibodies with FITC, a solution of antibody (1 mg/mL) in 0.5 mol/L sodium carbonate, pH 9.5, was added to a glass tube (12 × 75 mm) that had been precoated with 80 µg FITC.[14] The tube was rotated for one hour at room temperature, and FITC-labeled antibody was separated from free FITC by using a Sephadex G-25 column equilibrated with 0.05 mol/L Tris, 0.1 mol/L glycine, and 0.02% sodium azide, pH 7.4. The ratio of molecules of FITC bound per molecule of antibody (FITC:protein or F:P ratio) was calculated by measuring the absorbance at 280 and 495 nm.[15] The antibody solutions were diluted with an equal volume of Tris-glycine-azide buffer that contained 0.35% BSA and stored at 4°C until required. The FITC-labeled antibodies are stable for at least 6 months. Tab was first radioiodinated by using lactoperoxidase[6] (specific activity of 1055 cpm/ng) and then labeled with FITC (F:P ratio of 1.23:1). The F:P ratio of the S12 used in these experiments was 3.82:1.

Flow cytometry. An EPICS-C flow cytometer (Coulter Electronics, Hialeah, FL) was used to perform the flow cytometry. Excitation at 488 nm was achieved by using an argon ion laser, and emitted fluorescence was collected after passing through a 495-nm-long pass filter. From 10,000 to 50,000 platelets were analyzed with a linear scale in each sample. Day-to-day calibration of the flow cytometer was performed with FITC-labeled 2.0 µm carboxylate microspheres (Polysciences, Inc, Warrington, PA): first the laser power was set at 1,100 mW, the beam was aligned to achieve maximum log forward-angle light scatter, and then the voltage of the photomultiplier tube was adjusted to achieve a constant mean fluorescence value.

In the experiments with the doubly labeled Tab antibody, increasing concentrations of antibody were incubated with aliquots of washed platelets (5×10^7/mL) that had been fixed by 1% parafor-

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maldehyde.[6] With one aliquot, the number of molecules of antibody bound per platelet was determined by measuring the radioactivity associated with the platelets.[6] A second (20 μL) aliquot (containing 10^6 platelets) was added to 980 μL of phosphate-buffered saline with azide and was used to determine the amount of fluorescence associated with the platelets by flow cytometry. From these measurements plus the known F:P ratio of the Tab antibody, the number of molecules of FITC bound per platelet could be calculated and correlated with the arbitrary units of fluorescence recorded by the flow cytometer.[16]

In the experiments with FITC-S12, washed platelets (2×10^8/mL) were incubated with increasing concentrations of thrombin for ten minutes at 37°C. The platelets were fixed with 1% paraformaldehyde and diluted to 5×10^7/mL with Tris-glycine buffer.[6] An aliquot (250 μL) was incubated with FITC-S12 for 30 minutes at room temperature, and then 20 μL (10^6 platelets) were added to 980 μL PBS-azide for analysis by flow cytometry.

RESULTS

Calibration of the flow cytometer. Platelets incubated with subsaturating concentrations of doubly labeled Tab antibody demonstrated a linear relationship of bound fluorescence and bound ^{125}I (r = .99, n = 5). Under the conditions used in these experiments, it was calculated that for each unit of fluorescence recorded by the flow cytometer the number of antibody molecules bound per platelet was 600. From the F:P ratio of the Tab antibody (1.23:1), each unit of fluorescence intensity was equivalent to 738 molecules of FITC. Therefore in experiments using other FITC-labeled monoclonal antibodies the number of antibody molecules bound per platelet could be determined by multiplying the fluorescence intensity by 738 and dividing by the F:P ratio.

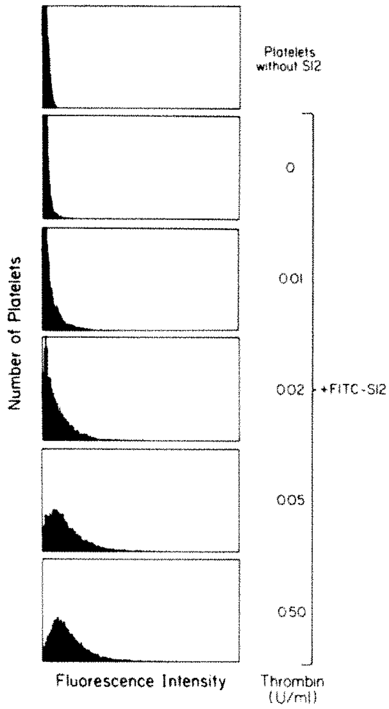

Fig 1. **Thrombin-stimulated exposure of GMP-140 on the platelet surface. Platelets were washed, treated with thrombin, fixed with paraformaldehyde, and reacted with FITC-S12. Platelets were analyzed in each sample for their fluorescence intensity with a linear scale. The top panel demonstrates the background fluorescence of washed platelets without FITC-labeled S12. The lower panels present the FITC-S12 binding results with control platelets and platelets treated with different thrombin concentrations. This figure illustrates a representative experiment.**

Table 1. Thrombin-Stimulated Exposure of GMP-140 on the Platelet Surface

Thrombin (U/mL)	Molecules GMP-140 per Platelet			
		Percentage of Platelets With		
	Mean	<2,000	>7,000	>10,000
0	1,120	93	1	0
0.0125	1,216	88	2	1
0.025	1,988	70	5	2
0.050	4,054	38	16	7
0.075	5,541	24	27	14
0.100	6,544	17	34	18
0.250	7,529	12	39	23
0.500	7,278	13	37	22

The data are the mean values for five experiments as illustrated in Fig 1. At each thrombin concentration the number of molecules of FITC-S12 bound per platelet was measured to determine the number of molecules of GMP-140 exposed on the cell surface. Data presented are the mean number of molecules of GMP-140 exposed per platelet for the total cell population and the percentage of platelets demonstrating different amounts of GMP-140 expression.

Platelet responses to thrombin. The pattern of platelet responses to thrombin as defined by the exposure of GMP-140 was determined by using FITC-S12 (Fig 1). The fluorescence associated with unstimulated platelets plus FITC-S12 was not different from the background fluorescence of washed platelets alone. At the lowest concentrations of thrombin examined, 5% of platelets became maximally acti-

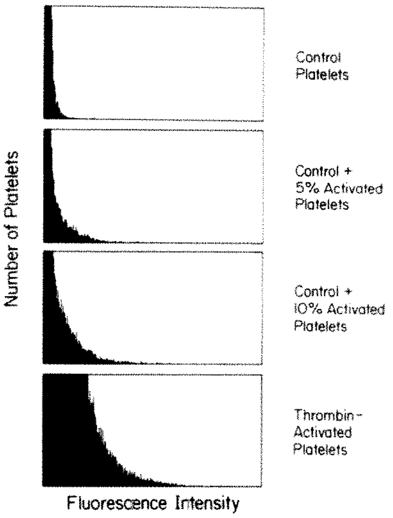

Fig 2. **Detection of thrombin-stimulated platelets mixed with unstimulated control platelets. Platelets were washed, and an aliquot was treated with 0.25 U/mL thrombin. The platelets were fixed with paraformaldehyde and reacted with FITC-S12. Platelets were analyzed in each sample for their fluorescence intensity with a linear scale, and the data printed with a histogram scale using greater magnification compared with Fig 1. The panels present the results with unstimulated platelets, with mixtures containing 5% and 10% thrombin-treated platelets, and with thrombin-stimulated platelets. This figure illustrates a representative experiment.**

vated (>7,000 molecules/platelet). At higher thrombin concentrations the number of activated platelets steadily increased and reached a maximum mean value of 7,529 molecules of S12 bound per platelet. Even at the thrombin concentration causing maximal stimulation (0.25 U/mL), 12% of platelets continued to bind less than 2,000 molecules of S12 per platelet (Table 1).

To demonstrate the sensitivity of the technique, experiments were performed to determine whether a small number of activated platelets could be identified within a population of unstimulated platelets (Fig 2). By using a greater magnification of the histogram scale compared with Fig 1, it was determined that as few as 5% activated platelets significantly altered the fluorescence profile. The increased fluorescence associated with the addition of 5% and 10% of activated platelets caused a slight but significant shift in the mean fluorescence intensity of the total platelet population ($P < .002$ and $P < .001$ (n = 9), respectively).

DISCUSSION

Flow cytometry represents an important advance in the ability to analyze individual platelets.[8-12] We have used flow cytometry with a calibration method that allows the accurate measurement of antibody molecules on the platelet surface[16] to define the heterogeneity of platelet secretion in response to thrombin. Platelet activation was assessed by using FITC-labeled S12, a monoclonal antibody to an α-granule membrane protein, GMP-140, that becomes incorporated diffusely across the surface plasma membrane during α-granule secretion.[2-7] The number of molecules of GMP-140 measured on the surface of stimulated platelets in this study was the same as we have previously measured by using ^{125}I-S12.[2,6] However, this study documents that within the normal population of circulating platelets some are exquisitely sensitive and respond to minimal thrombin concentrations with complete secretion, whereas other platelets failed to express GMP-140 even at the highest thrombin concentrations. These differences may represent heterogeneity of platelet α-granule content[17] or the effect of platelet aging in the circulation with a gradual loss of membrane responsiveness.[18]

In an earlier study we demonstrated that acutely ill patients with adult respiratory distress syndrome had circulating platelets that had undergone secretion in vivo, which was defined by the parallel appearance of GMP-140 and the α-granule–secreted protein thrombospondin on their membrane surface.[6] Since only the mean value for cell surface GMP-140 and thrombospondin could be measured, these observations represented a major abnormality. The quantitative analysis of platelet surface glycoproteins by fluorescence flow cytometry described in this study allows the detection of a small population of abnormal platelets and will provide a more accurate definition of acquired platelet membrane disorders.

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Monoclonal Antibody 7D5 Raised to Cytochrome b_{558} of Human Neutrophils: Immunocytochemical Detection of the Antigen in Peripheral Phagocytes of Normal Subjects, Patients With Chronic Granulomatous Disease, and Their Carrier Mothers

By Michio Nakamura, Masakazu Murakami, Toshihiko Koga, Yujiro Tanaka, and Shigeki Minakami

We have established a monoclonal hybridoma clone that produces IgG1 against the cytochrome b_{558} of human neutrophils. The antibody 7D5, secreted by the hybridoma, bound to solubilized cytochrome b of the neutrophils but not to other proteins such as hemoglobin, myeloperoxidase, and pig cytochrome P-450. Immunocytochemical studies of normal human peripheral blood showed that 7D5 bound to neutrophils and monocytes but not to lymphocytes or erythrocytes. The neutrophils of male patients but not of a female patient with chronic granulomatous disease lacked the antigen of 7D5 as well as the absorption spectrum for cytochrome b_{558}. A mosaic of the antigen-positive and -negative neutrophils was observed in mothers of the male patients. These biochemical and immunocytochemical results indicate that 7D5 is a specific antibody against cytochrome b_{558} of human phagocytes.

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PHAGOCYTE-SPECIFIC cytochrome b (cytochrome b_{558}) was first discovered in equine granulocytes[1] and was spectrophotometrically characterized in rabbit granulocytes by Shinagawa et al[2] in 1966. Segal and colleagues confirmed its presence in human neutrophils and called it cytochrome b_{-245} because they found that its redox potential (Em, 7.0) is −245 mV, exceptionally low for a b-type cytochrome.[3] Cytochrome has been suggested as an important component for the activation of oxygen during phagocytosis because the cytochrome was reduced under anaerobic conditions if the cells were stimulated[4] and it was not detected spectrophotometrically in the neutrophils of the majority of male patients with chronic granulomatous disease (CGD).[5] Convincing evidence for its role in phagocytosis-specific NADPH oxidase, however, has not yet been obtained, and the dispute over its molecular weight remains unsettled.

We tried to obtain a monoclonal antibody against the cytochrome in order to get definitive information on the hemoprotein. Because completely purified antigen was not necessary for obtaining monoclonal antibodies, we used a cytochrome b–rich fraction of human neutrophils without using any complicated purification procedure that seems to have led previous authors to propose various molecular weights ranging from 11,000 to 235,000 daltons.[6,7]

As the first report, we describe here a monoclonal antibody, 7D5, that binds specifically to the cytochrome of human neutrophils. The antigen of 7D5 has been immunocytochemically demonstrated to be present in normal neutrophils and monocytes but not in the cells of male patients with CGD. Mothers of the patients have two populations of neutrophils: one normally contains the antigen and the other does not.

MATERIALS AND METHODS

Materials. A mouse myeloma cell line, SP2/0, was kindly supplied by Dr K. Yagawa (Kyushu University, Japan). The monoclonal cell line that produces F4-11 (IgG1) against human carcinoembryonic antigen (hCEA) was generously provided by Dr M. Kuroki of Fukuoka University, Japan.[8] Polyclonal IgG1 was purified from nonimmune mouse sera by Affi-Gel protein A of Bio-Rad (Richmond, CA).[9] Purified cytochrome P-450 of pig liver was a gift from Dr Y. Yasukochi (Tokyo Medical and Dental College). All plasticware except filtration plates were Falcon products (Becton Dickinson, Oxnard, CA). The filtration plates, Millititer-HA, were purchased from Millipore Corp (Bedford, MA). Polyethylene glycol 6000 and aminopterin are the products of Wako Chemicals (Osaka, Japan). The MonoAb-Screen System, which contained β-galactosidase–conjugated antimouse IgG, was purchased from Zymed Laboratories, Inc (San Francisco). Vectastain, an avidin-biotin-peroxidase complex kit, was obtained from Vector Laboratories, Inc (Burlingame, CA).

Blood. Blood was drawn into anticoagulant-containing tubes from normal subjects, patients with CGD, and their mothers after their and/or their parents' informed consents were obtained.

Preparation of the cytochrome b–rich membrane fraction and solubilized cytochrome b. Human peripheral leukocytes were separated from acid-citrate-dextrose blood supplied by the Fukuoka Red Cross Blood Center according to the method previously described by Wakeyama et al.[10] The cytochrome b–rich fraction (C fraction) was prepared by the method of Gabig[11] without phagocytotic stimulation of the cells.

Phagocyte-specific cytochrome b was solubilized from the C fraction by 0.5% (wt/vol) Triton N-101 according to the method of Harper et al[12] in 3-[N-morpholino]propanesulfonic acid (Mops) buffer (50 mmol/L Mops, pH 7.0, 100 mmol/L KCl, 5 mmol/L β-mercaptoethanol) at 4°C. The concentrations of cytochrome b in the C fraction and in the solubilized preparation were about 16 nmol/mL and 3 nmol/mL, respectively.

Immunization. An inbred BALB/c mouse was immunized three times, each time with 0.2 mL of a mixture containing 0.1 mL of C fraction and 0.1 mL Freund's complete adjuvant at intervals of 3 weeks. Ten days after the final immunization, spleen cells were

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separated and immunized in vitro by solubilized cytochrome b (6 to 60 pmol with 10 to 100 μg of Triton N-101 in 10 mL culture medium) according to the method of Luben and Mohler.[13]

Hybridization and screening. At the fourth day of the in vitro immunization, spleen cells (5×10^8 cells) were harvested and hybridized by polyethylene glycol 6000 with logarithmically grown SP2/0 at a 6:1 ratio. The hybridized cells were washed once with RPMI 1640 (GIBCO, Grand Island, NY), suspended in medium containing 10% fetal calf serum, and seeded into 2,400 wells of 96-well microplates (Falcon 3072) at 2×10^5 spleen cells per well as reported by de St Groth and Scheidegger.[14] Eleven to 16 days after the seeding of cells, media of 1,645 wells with colonies resistant against hypoxanthine-aminopterin-thymidine were screened on 96-well filtration plates, Millititer HA, by an enzyme-linked immunosorbent assay with the MonoAb-Screen System. Each well of the plates had been coated with 25 μL of diluted C fraction that contained 2.9 pmol of cytochrome b. Each positive 499 colony was transferred to 24-well multiwell plates (Falcon 3047) and grown in 1.2 mL of medium. The second screening was carried out by an absorption assay of solubilized cytochrome b.

Absorption assay of cytochrome b. Each hybridoma medium (usually 1 mL) was incubated for one hour with 10 μL of Sepharose 4B affinity gels. The ligand of the gels is rabbit IgG, which is monospecific to mouse IgG (heavy and light chains). After washing the gels four times with phosphate-buffered saline (PBS) containing 1 mol/L NaCl and 0.1% Tween 20 and once with 0.1% Triton N-101–containing Mops buffer (25 mmol/L Mops, pH 7.0, 125 mmol/L KCl), the gels were incubated with 1.0 to 1.5 pmol of cytochrome b in 150 μL of the Mops buffer for 30 minutes at room temperature. After centrifugation of the gel suspension for four minutes at 10,000 g, 100 μL of the supernatant was transferred to a glass tube for the fluorometry of heme.

Fluorometric assay of heme. Heme in the supernatant was determined by the method of Morrison[15] with a slight modification described by Sassa.[16] To the supernatant, 0.4 mL of 2 mol/L oxalic acid was added, and the mixture was kept at 100°C for 30 minutes. After an addition of 0.7 mL of distilled water to the mixture, heme derivatives in the mixture were excited at 405 nm, and the consequent fluorescence emission was detected at 605 nm on a Hitachi 650-40 spectrofluorometer (Hitachi, Ltd, Tokyo).

Release of cytochrome b_{558} from affinity gels. The cytochrome b bound to affinity gels was washed once to six times (Fig 1) or four times (Fig 2) with 1 mL Mops buffer (25 mmol/L Mops, 125 mmol/L KCl, pH 7.0) containing Triton N-101 (0.01% or 0.4%) and 1 mol/L NaCl. The trapped cytochrome was released from the gels by the addition of 150 μL of 0.1 mol/L glycine buffer (pH 2.5) with 0.1% Triton N-101 or 0.25% *N*-octylglucoside. The cytochrome recovered from the supernatant after centrifuging the gel suspension was assayed as described earlier.

Preparation of peripheral phagocytes for immunocytochemistry. A neutrophil-rich fraction was prepared from the mixture of 4 vol of peripheral blood with 1 vol of 3% (wt/vol) polyvinylpyrrolidone as reported by Wakeyama et al.[10] A mononuclear cell fraction was prepared from whole blood on Conray-Ficoll as reported by Böyum.[17] The fractions were washed four times with PBS and suspended in HEPES-saline buffer (17 mmol/L HEPES, pH 7.4, 5 mmol/L glucose, 1 mmol/L $MgSO_4$, and 0.5 mmol/L $CaCl_2$). The concentration of phagocytes was adjusted to 0.3 to 1.0×10^7/mL.

Immunocytochemical demonstration of antigen of 7D5. Immunoperoxidase staining by the avidin-biotin-peroxidase complex was carried out as described by Hsu et al[18] with use of Vectastain. A drop of either cell fraction was put on a slide and incubated for 37°C for cell attachment. Unattached cells were rinsed from the glass with warm PBS. The remaining cells were air-dried, fixed in Hanks' balanced salt solution by 1% (wt/vol) paraformaldehyde and 0.02% (wt/vol) glutaraldehyde for 90 minutes at 4°C, and freeze-thawed once for penetration of the antigen through membranes. Nonspecific binding sites for immunoglobulin were blocked by the treatment of the cells with nonimmune horse serum for 20 minutes. About 100 μL of diluted culture medium containing monoclonal antibody 7D5 (4 μg/mL) was dropped on the glass and kept overnight in a refrigera-

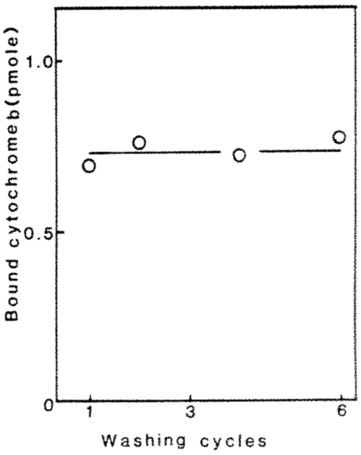

Fig 1. Demonstration of stable binding of solubilized cytochrome b to 7D5-treated affinity gels. Cytochrome b bound to 10 μL of the affinity gels was washed one to six times with 1.0 mL of Mops buffer containing 0.01% Triton N-101 and 1 mol/L NaCl and was released by glycine buffer (pH 2.5). The heme of the released cytochrome b was assayed as described in Materials and Methods.

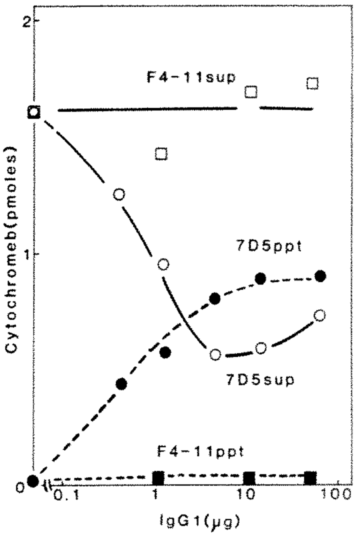

Fig 2. Dose responses of 7D5 (○, ●) and F4-11 (□, ■) media on cytochrome b–binding activity. A certain volume of 7D5 or F4-11 was mixed with 10 μL of antimouse Ig G-affinity gels. The mixture was centrifuged, and the gels that sedimented were incubated with solubilized cytochrome b. After centrifugation of the gel suspension, the cytochrome remaining unbound in supernatant (solid line) and the cytochrome specifically bound to gels via monoclonal antibody (broken line) were assayed as in Fig 1. Each point indicates the mean of two values. For details see Materials and Methods.

tor. After thorough washing with PBS, the cells were incubated with biotinylated horse antimouse IgG for one hour at room temperature. Endogenous peroxidase activity was denatured by the incubation of the cells in methanol with 0.01% H_2O_2 for 60 minutes at room temperature. They were thereafter incubated with the mixture of avidin and biotinylated peroxidase for 70 minutes and washed four times with PBS. Peroxidase activity was developed by treating the cells with 6 mmol/L diaminobenzidine and 0.005% H_2O_2 in 50 mmol/L Tris buffer (pH 7.6) for three to five minutes. The cells were counterstained by hematoxylin and embedded in paraffin. Photographs were taken using a Nikon FX-35A with a UFX-II exposure control unit on a Nikon XF-21 microscope.

Plain culture medium and F4-11, a monoclonal antibody against hCEA, were used for negative controls.

Statistical analysis. Means obtained from three or more values accompany the SD Student's *t* test was used for confidence analysis.

RESULTS AND DISCUSSION

We prepared a C fraction of Gabig[11] for in vivo immunization of a mouse and the solubilized preparation of the cytochome for in vitro immunization of spleen cells and for binding assays of the cytochrome to monoclonal antibodies. The reduced minus-oxidized difference spectrum of the samples has the same absorption peaks at 429 nm, 530 nm, and 558 nm and a trough at 414 nm (data not shown), consistent with those of the cytochrome b found in rabbit granulocytes,[2] porcine neutrophils,[19] human peripheral phagocytes,[3] and human colostral macrophages.[20] Therefore, we concluded that the cytochrome was phagocyte specific. The solubilized preparation did not contain any myeloperoxidase because no significant absorption peak was observed at 475 nm in its spectrum. All the heme in the solubilized preparation is therefore derived from the cytochrome b. This conclusion is further supported by the observation that the concentration of heme in the solubilized preparation (3.3 nmol/mL) was essentially the same as that of the cytochrome (3.0 nmol/mL) assayed spectrophotometrically using 21 as a millimolar extinction coefficient. These observations justified the use of a fluorometrical assay of heme for the detection of the cytochrome in the preparation.

The second screening of the antibodies was done by mixing monoclonal medium and the affinity gels ligated with rabbit IgG against mouse IgG, centrifuging the gels, and incubating them with the solubilized cytochrome b. After the gels had been treated with the medium containing 7D5, the heme remaining in the supernatant was 0.09 ± 0.14 pmol, about 15% of the applied cytochrome. On the other hand it was 0.68 ± 0.13 pmol when the gels had been treated with the medium containing monoclonal antibody against a different protein of the C fraction of Gabig. The hybridoma secreting 7D5 was cloned twice by limiting dilution, and the resultant monoclonal hybridoma was ascertained to secrete IgG1 (data not shown). The antibody 7D5, secreted by the cloned hybridoma cells, was used in the present investigation.

The monoclonal antibody 7D5, but not nonimmune polyclonal IgG1, absorbed solubilized cytochrome b_{558} (Table 1). The 7D5 therefore specifically bound to the cytochrome of human neutrophils. To exclude the possibility that 7D5 binds to the cytochrome through other protein in micelles, we extensively washed the gels with Mops buffer that contained 0.01% (Fig 1) or 0.4% Triton N-101. The cytochrome bound to 7D5-treated affinity gels was not removed at all by washing six times (Fig 1), which corresponded to more than a 20^6 dilution. Essentially the same results were obtained even if the concentration of the detergent in the washing buffer was increased to 0.4%. The amounts of the cytochrome recovered from the gels after washing once and six times with that buffer were 1.1 ± 0.1 pmol (n = 4) and 1.1 ± 0.2 pmol (n = 4), respectively. Therefore, 7D5 should have specifically recognized the cytochrome.

Table 1. Amounts of Hemoprotein Remaining in Supernatant After Separation From IgG1-Treated Affinity Gels

	Supernatant Heme (pmol)	
IgG1	Cytochrome b	Cytochrome P-450
Control IgG1	0.85 ± 0.20	0.65 ± 0.11
7D5	0.08 ± 0.06	0.47 ± 0.05

Each value is a mean ± SD obtained from three assays (see Materials and Methods).

The antigen released from the gels by 0.1 mol/L glycine (pH 2.4) gave a relatively broad band around 90 kilodaltons on sodium dodecylsulfate–polyacrylamide gel electrophoresis. Biochemical properties of the antigen will be presented in detail elsewhere.

The specificity of the antibody was shown further by the following experiments. (1) The antibody did not bind to phenobarbital-induced pig cytochrome P-450 ($P > .05$, Table 1). (2) The binding of 7D5 to the cytochrome was quantitative, as shown by the dose-response curve of the antibody (Fig 2). The cytochrome removed by 7D5 from the solubilized preparation was quantitatively recovered from the precipitate gels by acid treatments. When F4-11, a control monoclonal antibody against hCEA, was used, no cytochrome was removed from the supernatant or recovered from the precipitate gels. We therefore conclude that 7D5 is a monoclonal antibody against the phagocyte-specific cytochrome b of human neutrophils.

We used 7D5 for immunocytochemistry to demonstrate the presence of the antigen in human peripheral blood. The antigen was shown as brown deposits of polymerized diaminobenzidine in the cytoplasm of normal neutrophils (Fig 3A). Essentially the same deposits were also observed in monocytes (Fig 4A), in agreement with the presence of spectrophotometrically detectable cytochrome b.[21] The deposits were granulated and diffusely distributed in the cells and overlapped with nuclei, thus suggesting that the antigen is located on lysosomes and cytoplasmic membranes. Further analyses including electron microscopy are, however, required to determine definitive subcellular localization of the antigen. No deposit was found in lymphocytes (Fig 3A, arrows) or in erythrocytes (Fig 3A, arrowheads). The latter observation indicates that hemoglobin, a b-type hemoprotein, is not antigenic to 7D5. A control monoclonal antibody (F4-11) did not give any deposits (Fig 3B) because of the absence of its antigen in these cells (M. Kuroki, personal communications). We could not completely denature the

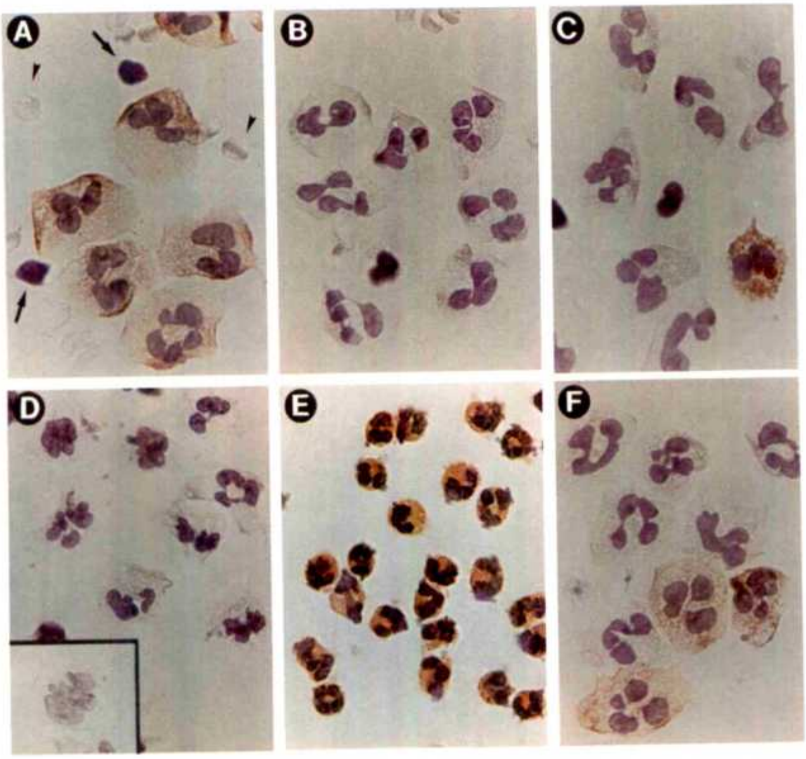

Fig 3. Immunocytochemical detection of the antigen of 7D5 in human peripheral neutrophils. (A) Neutrophils enriched from normal peripheral blood were attached to slides, fixed, freeze-thawed, incubated with culture medium containing 7D5, and finally counterstained by hematoxylin (for details see Materials and Methods). Arrows and arrowheads indicate lymphocytes and erythrocytes, respectively. (B) Same as A but immunostained with F4-11. (C) Same as A but immunostained with plain culture medium. A cell with brown deposits is an eosinophil that has been stained by residual endogenous peroxidase activity. (D) Neutrophils and a monocyte (inset) from a male patient with CGD were immunostained with 7D5 as in A. (E) Neutrophils from a female patient with CGD were immunostained with 7D5 as in A. Her cells were stained independently and look smaller than the others, possibly because of insufficient adhesion to glass. (F) Neutrophils from the mother of the patient shown in D were immunostained with 7D5 (Original magnification × 400).

endogenous peroxidase of eosinophils (Fig 3C) and failed to determine whether the cells had the antigen of 7D5.

The blood of patients with CGD and that of their mothers were also examined immunocytochemically. In contrast to normal phagocytes, neither neutrophils nor monocytes of a male patient were stained by 7D5 (Fig 3D). None of the cells was distinguishable from the normal phagocytes treated with culture medium (Fig 3C) or with F4-11 (Fig 3B). These two kinds of phagocytes of another five male patients (one reported in ref 22), were also not stained by 7D5. No characteristic spectrum of cytochrome b was observed in the neutrophils of those patients. Because myeloperoxidase was present in their cells, it should not have any antigenicity to 7D5. Neutrophils of a female patient with CGD were, however, clearly stained by 7D5 (Fig 3E). Her neutrophils exhibited a characteristic spectrum of the cytochrome (data not shown).

All mothers of the male patients thus far examined definitely had a mosaic of antigen-positive and -negative neutrophils in their blood. An example is shown in Fig 3F. This Lyon phenomenon indicates that the antigen is inherited on the X chromosome and is in good agreement with the findings that spectrophotometrically detectable cytochrome b is linked to that chromosome.[5] These biochemical and immunocytochemical results serve as two lines of confirmative evidence for 7D5 as a specific antibody against cytochrome b_{558} of human phagocytes.

The present observations suggest that the absence of the cytochrome b spectrum from the neutrophils of the six male patients with CGD means the absence of holoprotein of the

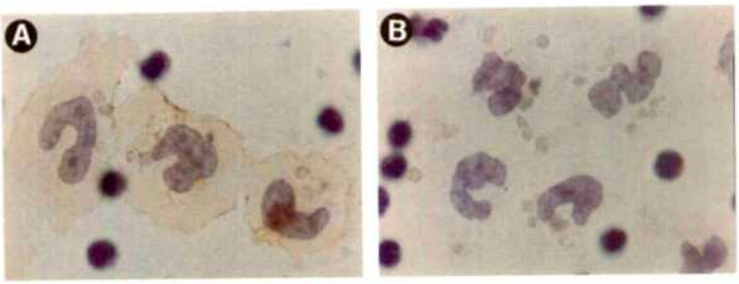

Fig 4. Immunocytochemical demonstration of the antigen of 7D5 in normal peripheral monocytes. Mononuclear cells separated from peripheral blood were attached to slides. The cells were treated as in Fig 3 and immunostained with 7D5 (A) or F4-11 (B). (Original magnification × 500.)

cytochrome but not the presence of abnormal apoprotein with the mutation at the site for binding heme. The abnormal apoprotein can exist only when it is stable and has either two mutations, one at the heme-binding site and the other at the antigenic site for 7D5, or a mutation at the common site for binding heme and 7D5. The former is unlikely because the two mutations hardly occur by chance in all six genetically unrelated patients. The latter is also unlikely because 7D5 failed to bind not only to polymorphonuclear leukocytes of rabbits, guinea pigs, and rats (data not shown) but also to other human hemoproteins. These results indicate that the antigen of 7D5 is different from evolutionally conserved structures such as heme-binding site.[23] The holoprotein of the cytochrome, therefore, must be absent in all neutrophils and monocytes of the six male patients with CGD and in all the antigen-negative cells of their mothers.

In summary, we obtained a monoclonal antibody, 7D5, that bound to phagocyte-specific cytochrome b. Our immunocytochemical studies with the antibody gave evidence for the lack of the cytochrome in peripheral neutrophils and monocytes of male but not of female patients with CGD and demonstrated the lyonization of the cytochrome in the cells of mothers of the male patients.

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Expression of the M-CSF (CSF-1) Gene by Human Monocytes

By Alessandro Rambaldi, Diane C. Young, and James D. Griffin

Monocyte colony-stimulating factor (M-CSF, CSF-1) is a macrophage lineage-specific growth factor. Northern blot analysis using a human M-CSF cDNA probe and a specific bioassay for human M-CSF were used to investigate the cellular sources of M-CSF. Expression of the M-CSF gene was induced in blood mononuclear cells stimulated by phorbol myristate acetate (PMA) or γ-interferon. When mononuclear cells were fractionated into highly purified populations of T cells and monocytes, M-CSF transcripts were detected predominantly in monocytes. Further, monocytes stimulated with PMA released an M-CSF-like activity. These results demonstrate that M-CSF can be produced by activated cells of the macrophage lineage.

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COLONY STIMULATING FACTORS (CSFs) are a group of glycoproteins that stimulate proliferation of hematopoietic precursor cells.[1] Four murine and human CSFs have been identified on the basis of different physical properties and target cell specificities. Multi-CSF or interleukin-3 (IL-3) has the capacity to stimulate proliferation of a broad range of progenitor cells including erythroid, granulocytic, mast cell, megakaryocytic, eosinophil, and multipotential progenitor cells.[2,3] GM-CSF stimulates granulocyte, monocyte, eosinophil, megakaryocyte, and erythroid colony formation.[1,4] G-CSF stimulates the formation of granulocytic colonies, and M-CSF (CSF-1) selectively stimulates the formation of monocyte colonies.[5,6] In addition, these factors may activate mature granulocytes and monocytes.[7-12]

Despite the importance of these factors in the regulation of hematopoiesis, little is known about the cell types that supply CSFs or the control of CSF gene expression. In this study, we describe the use of a cDNA probe for human M-CSF to investigate the expression of the M-CSF gene by highly purified populations of hematopoietic cells. A specific bioassay was used to detect human M-CSF activity separately from GM-CSF and G-CSF activity. The results indicate that unstimulated T lymphocytes and monocytes neither secrete M-CSF nor have detectable M-CSF transcripts. Exposure of monocytes but not T lymphocytes to γ-interferon (IFN) or phorbol myristate acetate (PMA) induces expression of the M-CSF gene and release of biologically active M-CSF. This suggests that monocytes, like T cells[13,14] and B cells,[15,16] can be induced to release growth factors specific for their own lineage.

MATERIALS AND METHODS

Human cells and cell lines. Peripheral blood cells were obtained from volunteer donors and mononuclear cell suspensions prepared by Ficoll-Hypaque density gradient centrifugation. T lymphocytes were prepared by E rosetting and were further purified by removal of any residual plastic-adhering cells. Monocytes were prepared by two different methods. In the first method, monocytes were purified from E cells by two plastic-adhering steps as previously described.[17] In the second method, B cells, T cells, and natural killer cells were removed from E cell fractions by immune rosettes using the lineage-specific monoclonal antibodies anti-B1, T11, and NKH1.[18] This method avoided adherence. The purity of all cell preparations was determined by immunofluorescence staining with the monoclonal antibodies anti-Mo2, -HLA-DR, -T11, -T3, -B1, and -NKH1.[19] All monocyte preparations used in this study contained less than 1% detectable T11+ or T3+ cells. All T cell preparations contained less than 1% detectable Mo2+ cells. Purified cells were cultured for various times at a concentration of 1×10^6/mL in RPMI 1640 with 10% fetal bovine serum (FBS), glutamine, and antibiotics (GIBCO, Grand Island, NY) at 37°C. The concentration of endotoxin in this medium was less than 0.5 ng/mL as determined by the limulus amebocyte assay. Where indicated, 500 U/mL recombinant γ-IFN (kindly provided by Dr Steven Wright, Biogen Research Corp, Cambridge, MA), PMA (10^{-10} mol/L; Sigma Chemical Co, St Louis), or recombinant GM-CSF (final dilution, 1:1000 of COS cell supernatant; kindly provided by Dr Steven Clark, Genetics Institute, Cambridge, MA)[7] were added to the culture media. The U937[20] and Hl-60[21] cell lines were obtained from the American Type Culture Collection, Rockville, MD, and cultured in RPMI 1640 medium supplemented with 10% FBS.

Preparation of total cellular RNA and Northern blot analysis. Total cellular RNA was isolated as previously described[22] by lysing cells in guanidium isothiocyanate followed by recovery of RNA by centrifugation through cesium chloride. Fifteen-microgram samples were then fractionated on a 1.2% agarose gel with 6% formaldehyde and blotted onto synthetic membranes (Gene Screen Plus, New England Nuclear, Boston). M-CSF mRNA was detected by using a 3.5-kb segment of the human M-CSF cDNA (CSF-1) inserted in the Xho site of the pXMT 2 vector (Gordon Wong, manuscript in preparation). This M-CSF probe was generously provided by Drs Gordon Wong and Steven Clark, Genetics Institute. The probe was labeled to a specific activity of 10^9 cpm/μg by using hexanucleotide primers and ^{32}P-dCTP.[23] Hybridization was performed at 60°C in a solution containing 1 mol/L NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 100 μg/mL salmon sperm DNA, and 1×10^5 cpm/mL labeled probe. The membrane was washed with 2x SSC/1% SDS for one hour at 65°C and 0.1x SSC at room temperature for one hour (1x SSC = 0.15 mol/L NaCl, 15 mmol/L sodium citrate, pH 7.0). The blots were then dried and used to expose Kodak Xomat x-ray film with intensifying screens. In one experiment, GM-CSF transcripts were detected with a cDNA probe for GM-CSF that was previously described[7] and supplied by Drs Wong and Clark. Reanalysis of a blot for the second probe[24] was done by washing the membrane in boiling water followed by rehybridization as described above.

Bioassay for human M-CSF. Supernatants were prepared by

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incubating purified cell populations in RPMI 1640 medium with 10% FBS at 1×10^6 cells/mL for 48 hours. Human M-CSF activity was detected by its ability to stimulate the growth of murine macrophage colonies in agar.[25] Murine cells were used as targets for two reasons: (1) human M-CSF stimulated much larger macrophage colonies in mouse bone marrow than it did from human bone marrow; and (2) human GM-CSF does not stimulate the growth of any mouse colonies, whereas human G-CSF stimulates the growth only of granulocyte colonies (see Results). Thus, stimulation of murine macrophage colony growth distinguishes human GM-, G-, and M-CSFs. Colonies were grown in a double-layer agar culture system previously described in detail.[26] Colonies were enumerated on day 7, fixed in situ, and stained for expression of chloroacetate esterase (granulocytes) and alphanaphthyl acetate esterase (monocytes).[27] Recombinant human CSFs were obtained from Dr Steven Clark, Genetics Institute, and were in the form of media conditioned by COS cells transfected with a vector containing a complete cDNA for GM-CSF,[7,28] G-CSF, or M-CSF. The titers were determined by a CFU-GM assay using human or mouse (BALB/c) light-density, nonadherent marrow cells. Control supernatant from COS cells conditioned by mock (vector-only) transfection contained neither stimulatory nor inhibitory activity for mouse or human marrow CFU-GM.

RESULTS

Induction of the M-CSF gene in human monocytes. Expression of M-CSF mRNA was investigated in peripheral blood cells (Fig 1). In an initial experiment, M-CSF transcripts[6] were detected in peripheral blood mononuclear cells stimulated with PMA (10^{-10} mol/L, 24 hours) but not in unstimulated mononuclear cells (Fig 1). To determine which cell type was expressing the M-CSF message, blood mononuclear cells were carefully fractionated into T lymphocytes (greater than 99% T11+ cells) and monocytes (greater than 95% Mo2+ cells). The cells were then cultured with control medium, γ-IFN (500 U/mL), or PMA (10^{-10} mol/L). M-CSF transcripts were induced in monocytes by either γ-IFN or PMA but not in T cells by either treatment. In order to be certain that stimulation of the purified T cells was sufficient, the blots shown in Fig 1 were washed and rehybridized with a cDNA probe for GM-CSF (a factor known to be secreted by activated T cells).[7,24] In contrast to M-CSF, expression of the GM-CSF gene was readily detected following treatment of T cells with PMA[24] (data not shown). To define the kinetics of induction of the GM-CSF message with γ-IFN, purified adherent monocytes were cultured with γ-IFN (500 U/mL) for 2, 5, 12, or 18 hours. Detection of M-CSF transcripts was maximal at five to 12 hours (Fig 2). These results suggest that expression of the M-CSF gene is inducible in human monocytes by exposure to the T cell lymphokine γ-IFN or by activation of protein kinase C by PMA.

To further demonstrate that cells of the monocytic lineage are able to express the M-CSF gene, we studied the U937 and HL-60 cell lines after induction of monocytic differentiation with several stimuli.[20,21] As shown in Fig 3, PMA-stimulated HL-60 cells expressed the M-CSF message.

Secretion of biologically active M-CSF by highly purified human monocytes. To determine whether the increased levels of M-CSF mRNA detected in monocytes following treatment with γ-INF or PMA were accompanied by

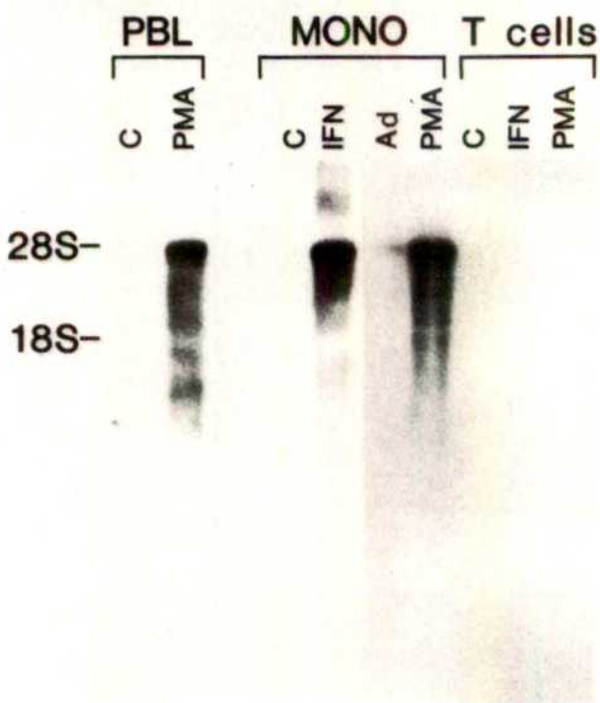

Fig 1. Northern blot analysis of M-CSF mRNA in human monocytes and T cells. Blood mononuclear cells (PBL) were fractionated into highly purified populations of monocytes (Mono) or T lymphocytes as described in Materials and Methods. In this experiment, monocytes were prepared by immune rosettes rather than adherence. The total cellular RNA was then isolated immediately (lane C) or after a 12-hour period of culture with γ-IFN, PMA (10^{-10} mol/L), or medium alone (AD indicates purified monocytes allowed to adhere in control medium for 12 hours). Fifteen-micrograms of RNA from each sample was fractionated on an agarose gel, transferred to a synthetic membrane, and hybridized to a ^{32}P-labeled cDNA probe for M-CSF.

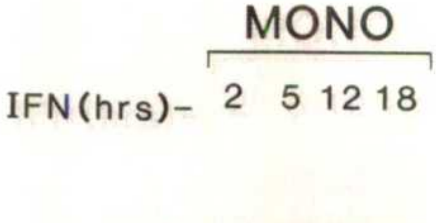

Fig 2. Monocytes isolated by adherence were cultured with γ-IFN for 2, 5, 12, or 18 hours prior to RNA extraction.

secretion of biologically active CSF activity, we cultured purified populations of monocytes and T cells either in control medium or medium containing γ-IFN (500 U/mL) or PMA (10^{-10} mol/L). In pilot experiments using recombinant human CSFs it was shown that rM-CSF stimulated the formation of only very small clusters of monocytes in agar culture of light-density human marrow cells. In contrast, GM-CSF stimulated the formation of large granulocyte and monocyte clusters and colonies, whereas G-CSF stimulated the formation of only granulocyte colonies. In mouse marrow, however, human rM-CSF stimulated the formation of large colonies of monocytes; rGM-CSF was inactive, and rG-CSF stimulated the formation of only granulocyte colonies (Table 1). Therefore, the activity of M-CSF could be distinguished from that of GM-CSF and G-CSF by using mouse marrow cells as targets and enumerating only monocyte colonies. Supernatants collected from unstimulated monocytes cultured for 48 hours contained a small amount of M-CSF activity (Table 1). Treatment of monocytes with g-IFN or PMA induced the release of approximately twofold and tenfold more M-CSF activity, respectively. M-CSF activity was not detectable in stimulated or unstimulated cultures of T cells. PMA also induced the release of a G-CSF–like activity from monocytes (Table 1).

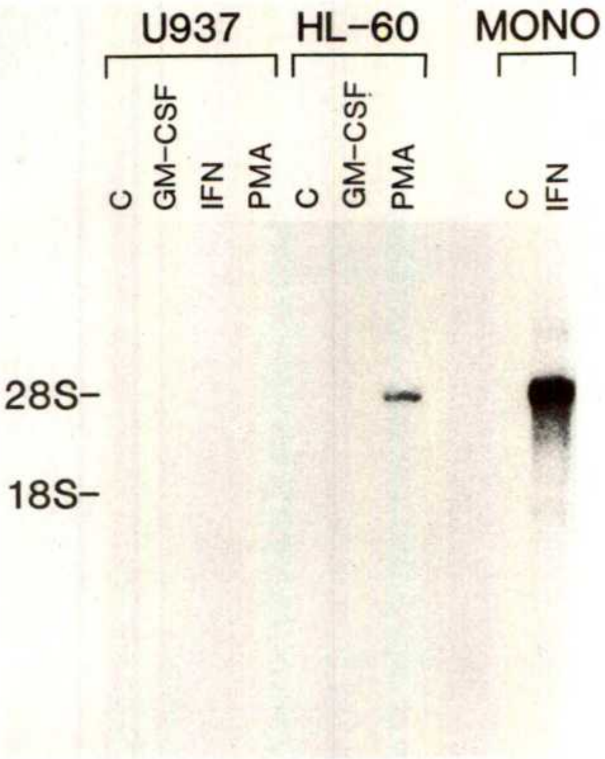

Fig 3. U937 and HL-60 cells were cultured at 1 × 10^6 cells/mL in control medium, GM-CSF (1:1,000 dilution), γ-IFN (500 U/mL), or PMA (10^{-10} mol/L) for 24 hours. The total cellular RNA was extracted as described in Fig 1 and expression of M-CSF transcripts determined. Monocytes purified by plastic adherence were similarly cultured with or without γ-IFN (500 U/mL, 14 hours) and assayed for M-CSF mRNA.

DISCUSSION

Human M-CSF (CSF-1) was the first of the human CSFs to be purified to homogeneity.[28,29] M-CSF from human urine is a glycoprotein of molecular weight 45,000 that has been shown to stimulate the growth of murine and human macrophage colonies.[1,30,31] A cDNA for human M-CSF has been isolated by using oligonucleotide probes based on the amino-terminus sequence for urinary CSF.[6] A full-length cDNA

Table 1. Secretion of M-CSF by Human Monocytes

	Murine CFU-GM/5 × 10^4		Marrow Cells	
	Monocyte		Granulocyte	
Source of Human CSF	Exp 1	Exp 2	Exp 1	Exp 2
Recombinant human CSFs*				
rM-CSF	145 ± 6	141 ± 5	13 ± 1	1 ± 1
rG-CSF	21 ± 6	4 ± 1	103 ± 9	92 ± 11
rGM-CSF	14 ± 2	2 ± 2	10 ± 1	3 ± 1
Mock-CSF	16 ± 5	1 ± 1	14 ± 4	1 ± 2
T cell (T)/monocyte (Mono) conditioned media				
Control	19 ± 5	0 ± 0	9 ± 3	0 ± 0
Mono-CM	37 ± 2	5 ± 3	25 ± 6	15 ± 4
Mono + PMA-CM	136 ± 6	35 ± 1	85 ± 6	27 ± 2
Mono + IFN-CM	63 ± 5	23 ± 1	19 ± 2	15 ± 4
T-CM	16 ± 3	0 ± 0	8 ± 1	0 ± 0
T + PMA-CM	19 ± 5	0 ± 0	7 ± 1	0 ± 0
T + IFN-CM	17 ± 2	0 ± 0	9 ± 1	0 ± 0
PMA (10^{-10} mol/L)	22 ± 4	4 ± 2	7 ± 3	6 ± 2
IFN (50 U/mL)	16 ± 1	0 ± 0	6 ± 1	0 ± 0

Abbreviations: Exp, experiment; NT, not tested.

*Each CSF has used as a 1:500 final dilution of medium conditioned by COS cells transfected with a CSF cDNA. These supernatants were previously titered on human (GM-, G-CSF) or mouse (M-CSF) marrow CFU-GM, and a concentration more than twice the maximum was used in these experiments. Murine CFU-GM were assayed in a double-layer agar system (see Materials and Methods).

†Purified cell populations were cultured at 1 × 10^6/mL for 48 hours and cell-free medium used as a stimulus for murine CFU-GM at a final concentration of 10% (data shown) and 1% (data not shown, revealing similar results in lesser magnitude).

has been obtained from a human pancreatic cancer cell line known to be able to secrete M-CSF.[6]

Cells capable of producing M-CSF in the human and mouse have not been extensively investigated. A number of murine tissues have been shown to be capable of making M-CSF including fibroblasts[30] and embryonic yoke sac.[32] Adherent, stromal cell lines established from murine long-term marrow cultures have been shown to secrete M-CSF.[33] Also, murine bone marrow cells transformed by the human *R-myc* oncogene have been shown to secrete M-CSF.[34]

The cellular sources of human M-CSF have not been investigated. This is at least partly due to the lack of highly specific assays for human M-CSF activity. In this study, we have used a human M-CSF cDNA to investigate the expression of the M-CSF gene in highly purified populations of T lymphocytes and monocytes. These cells were selected for study because preliminary experiments indicated that M-CSF transcipts could be detected following PMA stimulation of blood mononuclear cells and also because T lymphocytes and monocytes have been shown in previous studies to have potentially important effects in regulating many aspects of hematopoiesis and immune function. Although M-CSF transcripts were undetectable in unstimulated fresh monocytes, "activation" of these cells with PMA or γ-IFN induced M-CSF gene expression. With the conditions used in these experiments, M-CSF transcripts were not detected in unstimulated or stimulated purified T lymphocytes. It is possible that M-CSF expression can be induced by other types of T cell activation and further possible that monokines such as IL-1 may be required. Additional experiments will be required to determine whether the M-CSF gene can be expressed by normal T cells during other conditions of activation.

Although the exact role of M-CSF in human hematopoiesis is uncertain, the finding that M-CSF can be secreted by macrophage lineage cells in a regulated manner is of considerable interest. Human macrophage precursors proliferate in response to M-CSF alone, and it is possible that M-CSF acts synergistically with other CSFs in the production of human macrophage colonies. The recently described hemopoietin-1 isolated from the human bladder carcinoma cell line 5637 may be a factor of particular interest.[35]

The results presented here further suggest that the role of M-CSF in the immune response should be investigated. Recent evidence in the mouse has indicated that exposure of macrophages to M-CSF markedly influences the basal level of Fc receptor capacity and expression of the Mac-1 antigen and promotes subsequent activation by lymphokines such as γ-IFN.[10] Thus, M-CSF may stimulate selected monocyte functions in an autostimulatory manner. Finally, other cells of the immune system also secrete autostimulatory factors. T cells produce IL-2 in response to specific stimuli,[13,14] and normal and neoplastic B cells may produce B cell growth factor 1.[15,16] Thus, release of autostimulatory factors appears to be a common mechanism whereby immunocompetent cells augment certain aspects of the immune response.

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A Monoclonal Antibody Reacting With Human Basophils

By Michael P. Bodger, Gordon L. Mounsey, Jan Nelson, and Peter H. Fitzgerald

Bsp-1 is an IgM murine monoclonal antibody raised against the human erythroblastic leukemia cell line (HEL) that reacts with basophils but not neutrophils or eosinophils. Western blotting techniques showed that Bsp-1 reacts with a 45-kilodalton surface antigen on HEL cells. The distribution of Bsp-1 antigen on leukemic cells is confined to a basophilic leukemia cell line, KU812, chronic myeloid leukemia with basophilia, and some cases of acute undifferentiated leukemia. Bsp-1 might therefore be a useful reagent for the study of basophil function and differentiation.

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THE CLASSIFICATION of subsets of myeloid and lymphoid cells has been greatly aided by the generation of monoclonal antibodies (McAbs) that recognize lineage-specific cell surface antigens. McAb production has been generally achieved by immunizing mice with either purified normal cells, leukemic cells of a given lineage,[1-3] or leukemic cell lines that express multilineage cell markers.[4,5] Although several McAbs to granulocytic cells exist, only AHN-7[6] and EO-1[7] have documented reactivity with basophils. A detailed phenotypic analysis of the cell surface antigens on basophils has not been reported. This is partly due to the fact that basophils are the least common blood granulocyte and consequently their isolation in numbers sufficient for phenotyping has been technically difficult. A McAb that reacts with basophils but not other granulocytic cells would be useful for analyzing basophil function and differentiation. We report the generation of a McAb against a 45-kilodalton (kD) surface antigen on the human erythroleukemic (HEL) cell line[8] that reacts with basophils but not neutrophils or eosinophils.

MATERIALS AND METHODS

Preparation of McAb

A BALB/c mouse was immunized intraperitoneally with 1×10^7 HEL cells on days 1, 14, and 21, and cell fusion was performed on day 24 using procedures described elsewhere in detail.[9] The cells of one well reacting with HEL cells and rare mononuclear cells from bone marrow in a radioimmunoassay were selected for further study. The hybrid cells were cloned twice by limiting dilution, and ascites fluid was raised in pristane-primed BALB/c mice. The antibody was designated Bsp-1.

Preparation of Cells

Normal cells. Peripheral blood, umbilical cord blood, and bone marrow cells were separated by centrifugation on 60% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradients. Mononuclear cells were recovered from the interface, and granulocytes were recovered from the pellet after red cell lysis.[10] Sheep erythrocyte rosetting (E rosette+) cells and adherent cells were obtained from peripheral blood mononuclear cells.[11]

Normal basophils. Basophils were enriched from peripheral blood by using a modification of a previously described technique.[12] Blood was collected into EDTA, and buffy coat cells prepared from dextran-sedimented blood were layered over a multistep Percoll gradient of densities 1.070, 1.080, and 1.088 g/mL. The cells were centrifuged at 400 g for 25 minutes. A cell fraction enriched for basophils (mean, 22%) was collected from the 1.070/1.080 interface, and adherent cells were depleted overnight in RPMI containing 20% fetal calf serum (FCS) prior to staining with Bsp-1 antibody.

Human leukemic cell lines. Established human leukemic cell lines (listed in Table 3) were maintained as suspension cultures in 10% FCS/RPMI and tested for reactivity with Bsp-1 by using indirect immunofluorescence.[9] The erythroid cell lines OCI-M-1 and OCI-M-2[13] were assayed by Dr H.A. Messner (Ontario Cancer Institute, Toronto), and the basophilic cell line KU812[14] was assayed by Dr K. Kishi (Niigata, Japan).

Human leukemic cells. Leukemic cells from peripheral blood and bone marrow from patients with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), acute undifferentiated leukemia (AUL), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), and B cell lymphoma were tested for reactivity with Bsp-1 by using indirect immunofluorescence.

Reagents and Indirect Immunofluorescence

The following heterologous antisera and IgG class monoclonal antibodies (McAbs) were used in double combination with Bsp-1 to assess whether the antigens detected were present on Bsp-1+ cells: OKT3 (Ortho Pharmaceutical Corp, Raritan, NJ); CMRF-2, CMRF-4, and CMRF-10[15] (provided by Dr J. McKenzie, Christchurch Hospital, New Zealand); rabbit antiglycophorin A (provided by Dr C. Gahmberg, University of Helsinki); rabbit antiplatelet antiserum (provided by Dr J. Fraser, Wellington Clinical School, New Zealand); B3/25 (provided by Dr I. Trowbridge, Salk Institute, San Diego); W6/32[16] and CA-2[17] (provided by Dr J. Bodmer, Imperial Cancer Research Fund, London); WM-12 and WM-15[18] (provided by Dr K. Bradstock, Westmead Hospital, Sydney, Australia); My4, My7, and My9 (Coulter Immunology, Hialeah, FL); and RFB-1.[9] Cord blood cells were labeled with each McAb and second-layer goat antimouse IgG (Y and L chain specific) conjugated to tetraethyl-rhodamine isothiocyanate (G anti-M-IgG-TRITC; Tago Diagnostics, Inc, Burlingame, CA) or rabbit antisera and G antirabbit-Ig-TRITC; the cells were washed twice and then labeled with Bsp-1 and second layer G anti-M-IgM (μ chain specific) conjugated to fluorescein isothiocyanate (G anti-M-IgM-FITC). Cells were examined under a Leitz Dialux microscope with a 63-phase oil objective and IV/F epifluorescence condenser containing selective filters for FITC (green) and TRITC (red).

For cell sorting, peripheral blood cells from normal volunteers and patients with CML were labeled with Bsp-1 and stained with

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G(Fab)$_2$-anti-M-IgM-FITC (Tago) and separated into positive and negative cell fractions on a FACS IV (FACS System, Becton-Dickinson, Sunnyvale, CA).[19]

Culture-Derived Hemopoietic Cells

The mixed colony assay was used to generate erythroid cells, granulocytes, macrophages, eosinophils, and mixed lineage cells from their respective progenitors.[19] Colonies of each cell lineage were picked from the methylcellulose cultures, pooled, and washed twice in phosphate-buffered saline containing 5% FCS, stained with Bsp-1, and analyzed by using indirect immunofluorescence. The morphology of the cells in each pooled fraction was assessed by May-Grünwald-Giemsa staining.

Cytochemistry

Cell smears were prepared by using a cytocentrifuge (Shandon Southern Products Ltd, Cheshire, England) and stained with May-Grünwald-Giemsa, Sudan black, myeloperoxidase, nonspecific esterase, and acid phosphatase.[20] The metachromatic nature of the granules in basophils was confirmed by using 1% toluidine blue in methanol at pH 3.0[20] or alcian blue staining.[21]

Characterization of Bsp-1 Antigen

HEL and K562 cells were boiled in 0.5 mol/L Tris-HCl, pH 6.5, 2.4% sodium dodecyl sulphate (SDS), and membrane components were electrophoretically separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).[22] For Western blotting the proteins were electrophoretically transferred in 25 mmol/L Tris, pH 8.3, 0.19 mol/L glycine, 20% methanol onto nitrocellulose (Schleicher and Schull, Dassel, West Germany), which was then blocked with 3% casein in 10 mmol/L Tris-HCl, pH 7.4, and 0.9% NaCl, washed, and incubated with a 1:10 dilution of Bsp-1 ascitic fluid. Following further washing in Tris-buffered saline, the nitrocellulose was incubated with sheep antimouse biotin (Amersham International, Amersham, England) in 1% bovine serum albumin, Tris-buffered saline, and streptavidin-biotinylated horseradish peroxidase (Amersham International) and developed with 0.06% *o*-phenylenediamine (Sigma Chemical Co, St Louis) in 50 mmol/L Na_2HPO_4 and 25 mmol/L citric acid, pH 5.0. SDS molecular weight markers (Sigma) were stained with 0.1% amido black.

RESULTS

Selection of Bsp-1

The Bsp-1 antibody was initially selected for its reactivity with rare mononuclear cells in human bone marrow. Bsp-1 is an IgM class antibody determined on cells by indirect immunofluorescence by using antimouse subclass-specific antisera.[9] Bsp-1 is cytotoxic in the presence of human and rabbit complement in a complement-mediated cytotoxicity assay.[19]

Table 1. Reactivity of Bsp-1 With Normal Human Hemopoietic Cells

Cell Type (No. Tested)	Percent Reactivity*
Peripheral blood (6)	
Mononuclear cells†	<0.2
Granulocytes‡	0.2-1.0
E rosette + T cells	0
Adherent cells	0
Platelets	0
Erythrocytes	0
Eosinophils (3)§	0
Umbilical cord blood (10)	
Mononuclear cells	<0.5
Granulocytes	0.5-2.0
Bone marrow (5)	
Mononuclear cells	<0.2
Granulocytes	0.2-1.0
Culture-derived cells (4)	
Erythroid (BFU-E)	0
Granulocytes (CFU-G)	0
Macrophages (CFU-M)	0
Eosinophils (CFU-Eos)	0
Mixed lineages (CFU-Mix)	0

*Five hundred cells per sample counted using UV microscopy.

†Cells recovered from the interface after centrifugation on 60% Percoll gradients.

‡Cells recovered from the pellet after lysing red cells.

§A patient with a parasitic (microfilaria) lung disease and 77% circulating eosinophils and two patients with eosinophilia (20% and 28% circulating eosinophils).

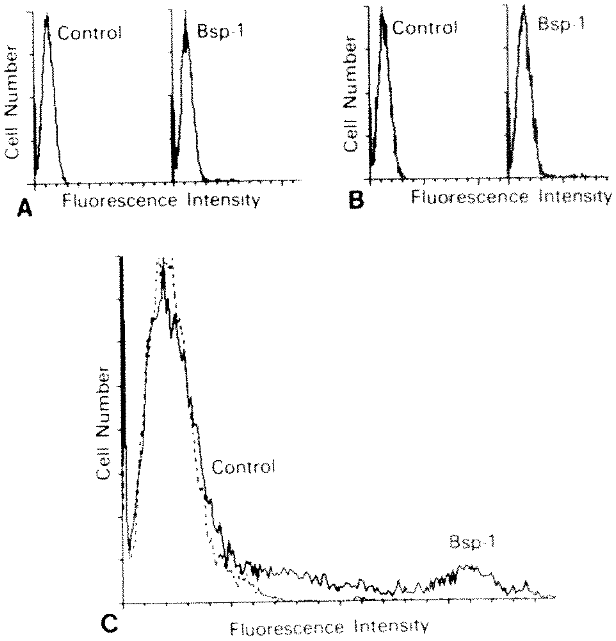

Fig 1. Bsp-1 antigen expression on human granulocytic cell populations. Cell pellets from Percoll density gradients were treated with NH_4Cl solution to lyse the red cells and were tested for antigen expression by indirect immunofluorescence using FACS IV analysis. Representative FACS histogram profiles are shown. (A) Peripheral blood granulocytes, 0.4% Bsp-1+ cells. (B) Cord blood granulocytes, 1.2% Bsp-1+ cells. (C) CML granulocytes, negative control (dashed line), Bsp-1 (solid line); 11.3% Bsp-1+ cells.

Reactivity of Bsp-1 With Normal Hemopoietic Cells

Mononuclear cells isolated from peripheral blood, cord blood, and bone marrow contained less than 0.5% Bsp-1+ cells by indirect immunofluorescence (Table 1). Granulocytic cells obtained from the cell pellet after red cell lysis contained up to 1% Bsp-1+ cells in peripheral blood and bone marrow and up to 2% Bsp-1+ cells in cord blood (Fig 1A and 1B). All other cells tested, including culture-derived hemopoietic cells, T cells, adherent cells, and eosinophils, were Bsp-1 −. Several attempts to sort Bsp-1 + from cord blood granulocytes were technically unsuccessful.

Antigenic Phenotype of Bsp-1 + Cells

Several IgG class McAbs and heterologous antisera against glycophorin A and platelets were used in combination with Bsp-1 to analyze the surface phenotype of Bsp-1 + cells in cord blood. The majority of Bsp-1 + cells exhibited HLA-ABC antigens identified by McAb W6/32 and CR3(C3bi) receptors identified by McAb WM-12 (Table 2). Bsp-1 + cells did not exhibit T cell antigens or myeloid antigens and were unreactive with antibodies to erythroid-specific glycophorin antigens, transferrin receptor, and platelets.

Reactivity of Bsp-1 With Hemopoietic Cell Lines

The reactivity of Bsp-1 antibody was tested against a panel of cultured human leukemic cell lines by using indirect immunofluorescence. Of four erythroid cell lines treated, only the immunizing cell line HEL was Bsp-1 + (Table 3). All myeloid and lymphoid cell lines tested were Bsp-1 −, as was the monocytic/histiocytic cell line U937. A basophilic cell line, KU812, derived from a CML patient in blast crisis had 10% to 15% strongly positive Bsp-1 + cells. A nonhemopoietic cell line, 5637, did not react with Bsp-1.

Reactivity of Bsp-1 With Hemopoietic Malignancies

The expression of Bsp-1 antigen was assessed on fresh leukemic cells. All cases of ALL, CLL, AML, and erythroleukemia tested were Bsp-1 − (Table 4). Two of nine cases of AUL contained 20% and 28% Bsp-1 + cells, respectively. Five cases of CML (chronic phase) tested contained Bsp-1 + cells. Cases 1 to 4 had 6%, 10%, 10%, and 8% Bsp-1 + cells respectively, and the fifth case of CML that presented with 35% Bsp-1 + cells showed an increase to 59% Bsp-1 + cells during the first week of treatment.

Table 2. Analysis of Bsp-1 + Cord Blood Cells With Combinations of Antisera

Antisera	Specificity	Percent Bsp-1 + Cells* Labeled
OKT3 (CD3)†	T cells	0
CMRF-4	Glycophorin A	0
Anti-glycophorin A‡	Glycophorin A	0
CMRF-10	Glycophorin C	0
Antiplatelet‡	Platelets	0
CMRF-2	Transferrin receptor	
B3/25	Transferrin receptor	0
My4 (CDw14)	Myeloid cells	0
My7 (CDw13)	Myeloid cells	0
My9	Myeloid cells	0
WM-15	Myeloid cells	0
WM-12 (CD11)	CR3 (C3bi) receptor	90
		70 strong positive
		20 weak positive
RFB-1	Progenitor cells	0
W6/32	HLA-ABC	75
		10 strong positive
		65 weak positive
CA-2	HLA-DR	0

*Two hundred Bsp-1 + cells per sample counted using UV microscopy.

†Clusters of differentiation.

‡Heterologous antisera produced in rabbits.

Table 3. Reactivity of Bsp-1 With Hemopoietic Cell Lines

Cell Line (Origin)	Percent Reactivity*
Erythroid	
HEL (erythroleukemia)	70-80
K562 (CML blast crisis)	0
OC1-M-1 (erythroleukemia)	0
OC1-M-2 (erythroleukemia)	0
Myeloid	
KG-1a (myeloblastic)	0
KG-1 (myeloblastic)	0
HL-60 (promyelocytic)	0
Monocytic/histiocytic	
U-937	0
Lymphoid	
CCRF-CEM (T cell)	0
Jurkat (T cell)	0
KM3 (T cell)	0
Molt 4 (T cell)	0
HUT 78 (T cell)	0
GH1 (T cell)	0
Nalm-6 (pre-B cell)	0
Raji (Burkitt's lymphoma)	0
Reh (null cell)	0
Daudi (B cell)	0
EBV-transformed PBL	0
Basophilic	
KU812	10-14
Nonhemopoietic	
5637 (bladder cell carcinoma)	0

Abbreviations: EBV, Epstein-Barr virus; PBL, peripheral blood lymphocytes.

*UV microscopy.

Bsp-1 + Cells in CML

Four of the five cases of CML had 6% to 12% basophilic cells at presentation, and the fifth case had 35% basophils. The percentage of Bsp-1 + cells in the cell pellet obtained after separation of CML cells on Percoll gradients was found to be similar to the percentage of basophils identified by May-Grünwald-Giemsa or toluidine blue staining. After sorting CML cells labeled with Bsp-1 (Fig 1C), virtually all the basophils were isolated in the Bsp-1 + fraction (Fig 2).

Table 4. Reactivity of Bsp-1 With Fresh Leukemic Cells

Classification	No. Positive/No. Tested*
T-ALL	0/1
Null ALL (CALLA +)	0/8
AUL (HLA-DR +, LCA +)	2/9
B-CLL (B1 +)	0/3
AML (M1-M5)	0/21
AML-M6 (erythroleukemia)	0/3
B cell lymphoma	0/1
CML	5/5

*UV microscopy.

Abbreviations: CALLA, common acute lymphoblastic leukemia antigen; LCA, leukocyte common antigen.

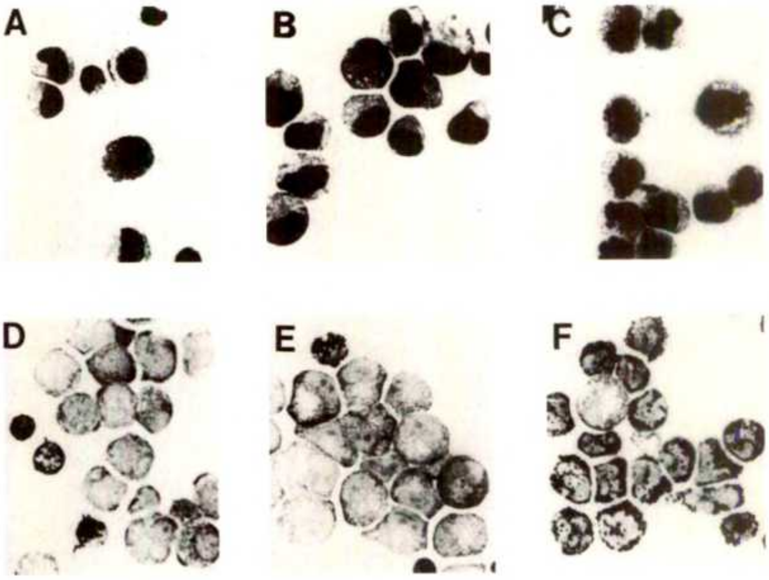

Fig 2. Cytochemical staining of CML cells separated into Bsp-1+ and Bsp-1− cell fractions on the FACS IV. May-Grünwald-Giemsa staining of (A) unseparated cells, (B) Bsp-1− cells, and (C) Bsp-1+ cells. Toluidine blue staining of (D) unseparated cells, (E) Bsp-1− cells, and (F) Bsp-1+ cells. Bsp-1+ cells are toluidine blue–staining basophils.

Bsp-1+ cells stained positively with toluidine blue (Fig 2) but were not stained with Sudan black, myeloperoxidase, nonspecific esterase, and acid phosphatase.

Biochemical Characterization of the Bsp-1 Antigen

Western blotting techniques revealed a major band at 45 kD and a minor band at 80 kD on HEL cells under reducing (Fig 3, lane C) and nonreducing (Fig 3, lane D) conditions. These bands were not detected on Bsp-1− K562 cells. In a further control, 29-kD and 32-kD bands identified by McAb RFB-1 were not detected on RFB-1− HEL cells.

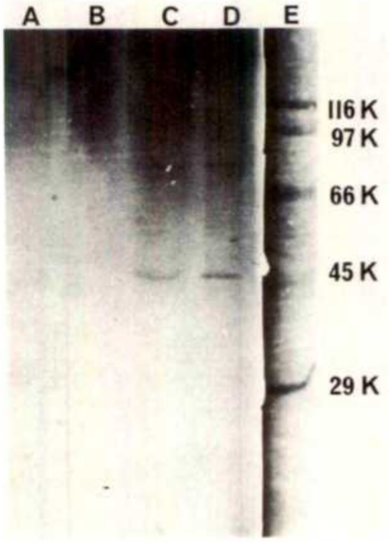

Fig 3. Western blotting detection of the Bsp-1 antigen. Lanes A and B are K562 cells under reducing and nonreducing conditions respectively. Lanes C and D are HEL cells under reducing and nonreducing conditions. The following molecular weight protein standards are shown in lane E; galactosidase (116k), phosphorylase B (97k), bovine albumin (66k), ovalbumin (45k), and carbonic anhydrase (29k).

DISCUSSION

We have derived a McAb, Bsp-1, against human basophils by using the human erythroleukemia cell line HEL as an immunogen. Bsp-1 was initially selected for its lack of reactivity on other hemopoietic cell lines and the very low percentage of positive cells observed in peripheral blood and bone marrow.

Of all the other cell lines tested, the Bsp-1 antigen was only detected on a subset of cells from the basophilic cell line KU812. Spontaneous differentiation of KU812 has decreased since its derivation, and only a low percentage of cells contain basophilic granules (K. Kishi, personal communication, May 1986). The percentage of Bsp-1+ cells in KU812 is consistent with the percentage of cells with basophil morphology. Excluding CML, only two fresh leukemias have presented with Bsp-1+ cells. Both examples were classified as acute undifferentiated leukemias and showed no evidence of basophilic maturation.

The close correlation between the percentage of basophils and Bsp-1+ cells in the peripheral blood of CML patients first suggested that Bsp-1 was reacting with basophils. This was confirmed by cell sorting and the recovery of greater than 95% of basophils in the Bsp-1+ cell fractions from normal and CML peripheral blood. Other blood granulocytes, including eosinophils, neutrophils, as well as monocytes, were unreactive with Bsp-1. To our knowledge, Bsp-1 represents the first antihuman basophil-specific McAb. In a recent study reporting the production of several McAbs using HEL as an immunogen, one antibody (53/10) reacted only with HEL cells from a panel of leukemic cell lines and was expressed on only a small proportion of bone marrow cells that did not include erythroid cells or granulocytes.[5] However, in this study basophils were not identified, and as no biochemical data on the 53/10 cell surface antigen were reported, the relationship between Bsp-1 and 53/10 remains unknown.

Cells derived from hemopoietic progenitor cells in the mixed colony assay did not express Bsp-1. Recently, pure and mixed basophil colonies in cultures of peripheral blood and bone marrow have been identified by using a modification of the methylcellulose technique.[23] Complement treatment of bone marrow or cord blood cells labeled with Bsp-1 does not eliminate myeloid, erythroid, or multipotential progenitor cell growth in the mixed colony assay (unpublished observations). However, we have as yet to establish whether our culture conditions support the growth of basophil progenitor cells and whether basophil progenitor cells express the Bsp-1 antigen. The fact that Bsp-1 reacts with circulating basophils in the peripheral blood and KU812 cells indicates that the Bsp-1 antigen is expressed on mature cells and a proportion of blast cells of the basophil lineage.

Bsp-1+ cells react with antibodies that bind to CR3 receptors and HLA-ABC antigens. Although Bsp-1 and HLA-ABC antibodies identify surface antigens of similar molecular weight, the vastly different cellular distribution of HLA-ABC antigens compared with the Bsp-1 antigen makes it unlikely that Bsp-1 is against an epitope on HLA-ABC antigens. The specificity of Bsp-1 for basophils makes this antibody a useful reagent for isolating basophils from hemopoietic tissue, studying basophil differentiation, and detecting basophilia in myeloproliferative disorders where basophils can present with abnormal morphology.[24]

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Type IIA von Willebrand Disease With Apparent Recessive Inheritance

By Akio Asakura, Janet Harrison, Edward Gomperts, and Charles Abildgaard

Type IIA von Willebrand's Disease (vWD) is the most common type II variant, and all reported cases (56 individuals in 26 families) have had autosomal dominant inheritance. An eight-year-old female with an increased bleeding tendency since infancy was found to have laboratory values typical of type IIA vWD, but her parents and siblings were asymptomatic. With the exception of uniformly decreased levels of ristocetin cofactor in relation to von Willebrand factor antigen, the results of family studies were normal including the presence of large multimeric forms of von Willebrand factor antigen. These findings are consistent with the propositus having the homozygous state of an autosomal recessive trait. Desmopressin infusion in the propositus was followed by a significant increase of factor VIII coagulant and von Willebrand factor antigen but a limited change in ristocetin cofactor with no development of large multimers.

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TYPE II VON WILLEBRAND'S disease (vWD), defined by the absence or decrease of large multimers of von Willebrand factor antigen (vWF:Ag), has been divided into a number of subtypes, the most common being type IIA. In addition to the absence of large multimers, type IIA vWD is characterized by (1) a disparity in the amount of vWF:Ag and vWF activity as measured by ristocetin (RCo), with vWF:Ag greater than RCo; (2) the presence of a repeating five-band pattern of small multimers seen on 3.0% agarose gel electrophoresis, with relative increased density of the outermost flanking subbands as compared with normal; (3) normal or decreased RCo-induced platelet aggregation; and (4) little or no response to infusion of desmopressin.[1] The inheritance of type IIA vWD has been dominant in 56 reported individuals in 26 families.[1-5] In this report, we describe an individual with type IIA vWD with apparent autosomal recessive inheritance.

CASE REPORT

The propositus is an eight-year-old white female with history of an increased bleeding tendency since infancy. At the age of 2 months she bled for a day following her first immunization injection, and she was noted to have easy bruising following minor injuries. At the age of 2 years, she was seen in the emergency room because of four days of bleeding from a tongue laceration. The diagnosis of vWD was made at this time based on the prolonged bleeding time and decreased levels of factor VIII coagulant (46%), vWF:Ag (40%), and RCo (<1%). Two-dimensional crossed-immunoelectrophoresis of vWF:Ag revealed an abnormal fast-moving component. The child subsequently has had repeated bleeding episodes following minor injuries and has received cryoprecipitate for treatment of a laceration and for a tooth extraction. More recently she received antifibrinolytic therapy and an infusion of desmopressin in preparation for a tooth extraction, and hemostasis was satisfactory.

Her family history is negative for bleeding manifestations. Both parents and three siblings (6, 14, and 16 years) are living and well, and all members of the family were evaluated.

Table 1. Values for VIIIC, vWF:Ag, and RCo for the Propositus and Family Members

	VIIIC (%)	vWF:Ag (%)	RCo (%)	RCo/vWF:Ag
Propositus	58	22	3	.14
Mother	144	89	42	.47
Father	100	73	44	.60
Brother (1)	100	107	53	.49
Brother (2)	120	95	58	.61
Sister	98	73	49	.67
Normal	100	100	100	1.02*
Normal range	50-150	50-150	50-150	0.72-1.59

*Mean ratio of 26 normal individuals.

MATERIALS AND METHODS

Computations of bleeding times were performed by the Ivy method. Blood collection, plasma preparation and storage, as well as methods for factor VIII coagulant activity (one-stage partial thromboplastin time method), vWF:Ag (Laurell), multimer analysis (sodium dodecyl sulfate agarose gel electrophoresis using 1.5% and 3.0% gels), and RCo (macroscopic agglutination technique using formalin-fixed platelets) have been described previously.[6,7] Ristocetin-induced platelet aggregation (RIPA) was measured by the method of Weiss et al.[8]

RESULTS

The results of plasma studies of the propositus, her parents, and three siblings are shown in Table 1. The propositus had a prolonged bleeding time (>20 minutes), significantly decreased vWF:Ag (22%) and RCo (3%), and normal RIPA. The only abnormality noted in the parents and siblings was a uniformly decreased amount of RCo in relation to vWF:Ag. The ratios of RCo to vWF:Ag for the parents and siblings were 0.47, 0.60, 0.49, 0.61, and 0.67 (mean, 0.57). For comparison, mean ratios of RCo to vWF:Ag in normal individuals and other vWD subjects studied in our laboratory are as follows: normals (26), 1.02; type I vWD (15), 1.21; type IIA (14), 0.51, and type IIB (9), 1.19.

The multimer patterns of vWF:Ag on 1.5% and 3.0% agarose gel electrophoresis are shown in Fig 1A and B. On 1.5% gel the propositus lacks large and intermediate multimers, whereas her parents and siblings all have normal patterns.

On 3.0% gel the propositus has a repeating five-band pattern of small multimers with a relative increase in the density of the outermost flanking subbands. The subband patterns of the parents and siblings all appear normal.

Infusion of desmopressin (0.3 μg/kg) into the propositus

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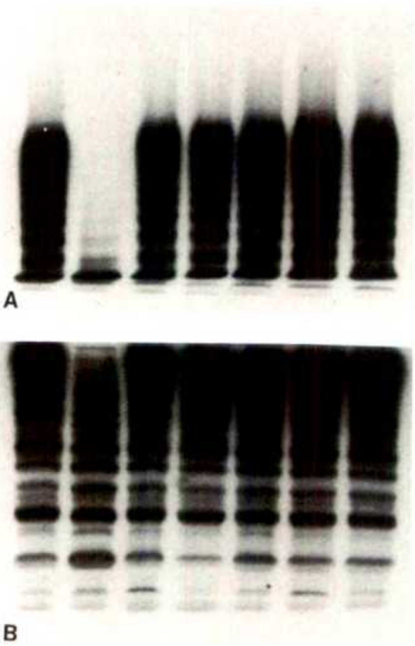

Fig 1. SDS agarose gel electrophoresis of vWF:Ag on 1.5% (A) and 3.0% (B) gels. From right to left the lanes are normal plasma, propositus, mother, father, brother 1, brother 2, and sister. On 1.5% gel, the propositus lacks large and intermediate multimers, whereas the other family members have normal patterns. On 3.0% gel, the propositus has a repeating five-band pattern of small multimers with an increase in density of the outermost flanking subbands of the small multimers, and other family members have normal patterns.

resulted in increases of factor VIII coagulant activity (18% to 128%) and vWF:Ag (23% to 114%), whereas RCo only increased from 3% to 17%. Plasma vWF:Ag multimers were analyzed before and after desmopressin infusion in the propositus; patterns are shown in Fig 2. On 1.5% gel only a few of the smaller intermediate multimers appeared following desmopressin infusion. An increase in density of the smallest multimers was noted on 3.0% gel.

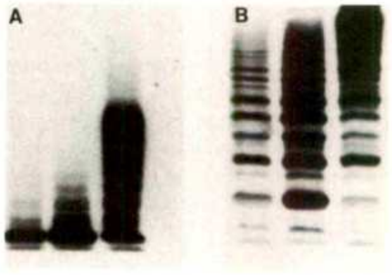

Fig 2. Before and after desmopressin infusion studies of the propositus plasma on (A) 1.5% and (B) 3.0% agarose gels. On 1.5% gel, the postinfusion sample in the center lane reveals a slight increase of intermediate multimers (normal plasma on the right). On 3.0% gel, an increased density of small multimer bands is seen in the postinfusion sample in the center lane (normal plasma on the right).

DISCUSSION

The laboratory findings related to the vWF in the propositus are similar to previously reported examples of type IIA vWD. In contrast to previous patients, the inheritance in the present case appears to be autosomal recessive rather than dominant. This interpretation is supported by the lack of bleeding manifestations and laboratory abnormalities in the parents and siblings of the propositus. This disparity in the amount of RCo to vWF:Ag in each of the asymptomatic family members is of interest and is similar to that observed in most type IIA patients with dominant inheritance. This observation suggests that each of the parents and siblings is heterozygous for this type of vWD variant abnormality and that the propositus is homozygous.

Although the propositus did not have abnormal bleeding following dental extraction performed after desmopressin infusion, she also received antifibrinolytic therapy at the time. Since the latter may have maintained hemostasis, the efficacy of desmopressin in the propositus is difficult to determine from this experience. There has been no further opportunity to administer desmopressin to determine whether there is an effect on the bleeding time or clinical bleeding manifestations in this patient.

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Factor XII Gene Alteration in Hageman Trait Detected by *TaqI* Restriction Enzyme

By F. Bernardi, G. Marchetti, P. Patracchini, L. del Senno, M. Tripodi, A. Fantoni, S. Bartolai, F. Vannini, L. Felloni, L. Rossi, F. Panicucci, and F. Conconi

A cDNA for coagulation factor XII has been used to investigate the presence of gene lesions and restriction fragment length polymorphisms in two brothers with Hageman trait and their family. A *TaqI* polymorphic fragment has been found in the two propositi and in 11 members of the paternal lineage. This polymorphism, absent in the normal population, is correlated with the reduction of factor XII activity and enables the identification of heterozygous factor XII deficiency. Factor XII gene deletion as the cause of Hageman trait in this family has been excluded. A restriction map has been constructed, and the *TaqI* polymorphic site has been localized within the 5′ portion of the gene. The mutation in the polymorphic site is probably the cause of the factor XII deficiency. Data suggest the presence of one factor XII gene per haploid genome.

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BLOOD COAGULATION factor XII or Hageman factor (FXII), a glycoprotein with a single polypeptide chain of molecular weight 80,000,[1] circulates in plasma as an inactive proenzyme; when activated, it acts as a serine protease in the initiation of the blood clotting cascade, kinin production, and fibrinolysis.[2] Hageman trait is an asymptomatic anomaly of in vitro blood coagulation due to a hereditary deficiency of FXII[3]; its diagnosis is based on finding a low plasma activity and antigen of the factor in clotting and immunologic assays. Since Ratnoff's[4] first report (1954), hundreds of cases have been observed. The anomaly is transmitted as an autosomal recessive character that in homozygotes (Hageman trait) causes a complete deficiency of FXII activity[3] and in heterozygotes, a partial deficiency of activity. Due to an apparent bimodal distribution, Veltkamp et al[5] inferred that at least two allelomorphic genes control the synthesis of the factor. A different form of Hageman trait with autosomal dominant inheritance has also been reported.[6]

The recent cloning of cDNA for FXII[7,8] enables the investigation of gene lesions and of restriction fragment length polymorphisms (RFLPs) at the FXII locus, useful for tracing the inheritance of Hageman trait and for genetic linkage studies.

In this paper a FXII *TaqI* RFLP, detected in two brothers carrying Hageman trait and in 11 members of their paternal lineage, is reported. This RFLP has been mapped within the FXII gene and correlated with the FXII plasma activities of the family members.

MATERIALS AND METHODS

Factor XII clotting activity and antigen determinations. FXII activity was determined by the activated partial thromboplastin time method using an appropriate deficient plasma substrate.[9] FXII antigen was estimated by quantitative immunoelectrophoresis according to Laurell's technique.[9] A monospecific heterologous antibody has been used (Behringwerke AG, Marburg, FRG).

Southern blot hybridization. Blood samples from 35 normal unrelated subjects from different Italian regions and from 24 members of the family under study were obtained by standard procedures.

DNA isolation and the Southern blotting technique were performed as previously described.[10] Six to 10 μg of DNA were digested with restriction endonucleases (Boehringer Mannheim, FRG; New England Biolabs, Boston).

FXII cDNA probes were obtained from a human liver cDNA library in the expression vector pEX1.[8] All experiments involving recombinant DNA were performed according to the accepted safety guidelines. Hybridization conditions (50% formamide and 10% dextran sulfate at 42°C) and washing of the filters were in accordance with the gene screen transfer membrane method (New England Nuclear).

RESULTS

Coagulation and immunologic assays. The hemostatic study of the two brothers with Hageman trait showed a markedly prolonged activated partial thromboplastin time and normal bleeding and prothrombin and thrombin times. Specific assays for coagulation factors showed a severe deficiency of FXII clotting activity (XII: C < 1 U/dL) and a lack of factor XII antigen (XII: Ag < 10 U/dL).

RFLP detection. In order to investigate gene alterations of RFLPs of the FXII gene, DNA from the two brothers with Hageman trait (IV-1 and IV-2 of the pedigree in Fig 1) and from their parents was digested with several restriction enzymes and hybridized to cDNA probes for FXII.

The probes have been isolated from a human liver cDNA library (pEX1 expression vector) by immunoblotting to an antibody specific for FXII. The DNA sequence of the inserts indicates that the partial cDNAs span from the leader peptide to aminoacid 79 (probe 1) and from aminoacid 121 to 396 (probe 2) of plasma FXII.[8]

In the *TaqI* digests hybridized to probe 2 (Fig 2A) two bands of 3.3 and 1.1 kb were found in the four subjects as well as in the 35 unrelated normal individuals examined. An additional 2.1-kb band was found in the two brothers and

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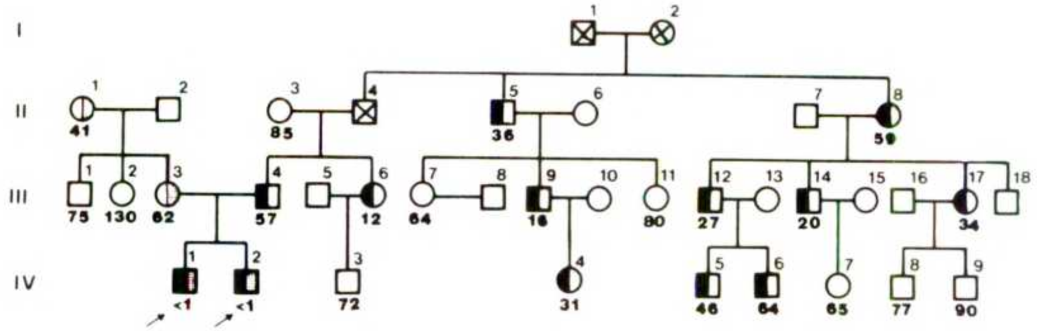

Fig 1. Pedigree of the Hageman trait family. ◐, presence of *Taq*I polymorphism; ⦶, FXII deficiency carried in the maternal lineage. FXII activity (FXII:C), when determined, is reported below each member of the family. The arrows indicate the two Hageman trait subjects.

their father. The polymorphic band (Fig 2B) was also detected in hybridizations with the 5′ cDNA probe 1.

Family studies. The inheritance of the *Taq*I RFLP and the FXII activity have been studied in the family of the two Hageman trait subjects.

Figure 1 shows the pedigree of the family including the results of the RFLP analysis and of the FXII activity. The *Taq*I RFLP is present in addition to the Hageman trait subjects in seven males and four females, all of the paternal lineage; intermediate FXII activity levels are present in members of both the paternal and maternal lineages.

The levels of FXII activity in polymorphic and nonpolymorphic subjects of the paternal lineage together with 15 normal control values are reported in Fig 3; activities are lower in the polymorphic subjects.

RFLP characterization and localization. Additional studies have been performed to determine the origin and to localize in the FXII gene the *Taq*I RFLP.

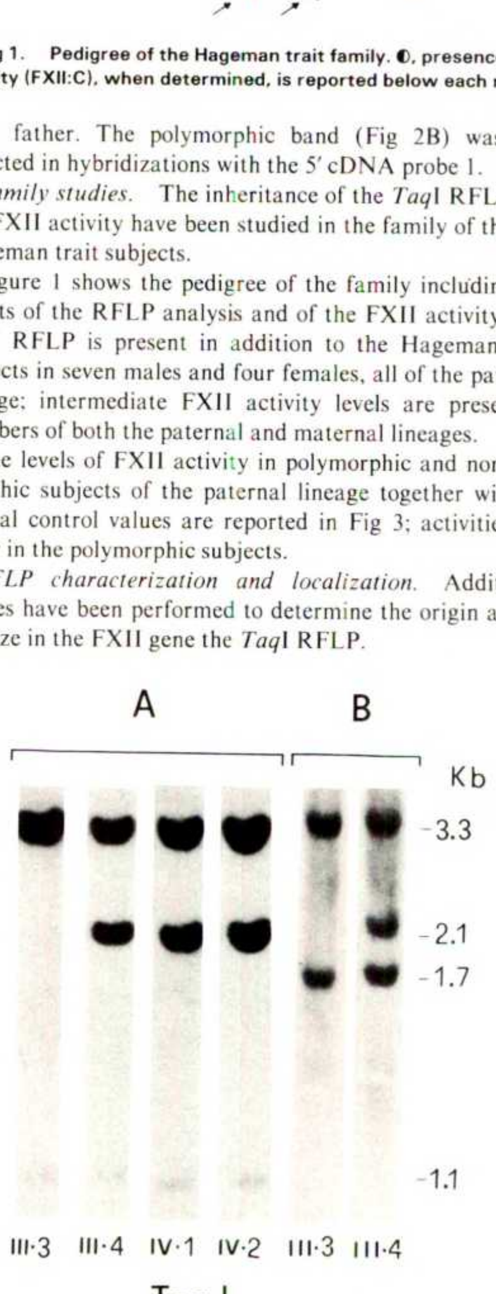

Fig 2. *Taq*I RFLP detection. DNA samples from Hageman trait subjects (IV-1, IV-2) and their parents (III-3, III-4) have been digested with *Taq*I (A and B). Southern blots were hybridized to a 3′ FXII cDNA (A) and to a 5′ FXII cDNA (B).

The intensity of the 3.3-kb band, using both cDNA probes, was reduced to half when the 2.1-kb band was present (data not shown), which suggests that the polymorphic band originates from the 3.3-kb band; thus two FXII genes should be present in the human diploid genome. Hybridizations of *Bam*HI and *Bgl*II DNA digests with both probes (Fig 4) produced large FXII bands common to normal and polymorphic subjects, thus excluding that the polymorphism originates from a gene deletion detectable by Southern blot analysis. The polymorphic pattern is therefore explained by an additional *Taq*I site cleaving the 3.3-kb DNA fragment in the 2.1-kb band and in a 1.2-kb band not hybridizing to the cDNA probes used. This 1.2-kb DNA fragment is probably an intronic sequence.

In order to localize the *Taq*I polymorphic site in the FXII gene, double digestions with several restriction enzymes were performed. In Fig 5A *Nco*I/*Taq*I and *Bcl*I/*Taq*I double-digestion patterns are reported. Two polymorphic bands of 0.47-kb (*Bcl*I/*Taq*I) and of 1.2-kb (*Nco*I/*Taq*I) are present in a *Taq*I polymorphic subject. Analysis of the length of the DNA fragments obtained with additional double digestions (*Taq*I/*Bgl*II, *Hind*III, *Pst*I, *Pvu*II, *Rsa*I) has located the *Taq*I polymorphic site in the 5′ portion of the FXII gene (Fig 5B). Exonic sequences detected by the 5′ FXII cDNA are

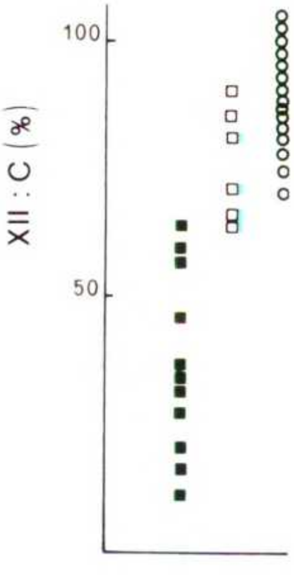

Fig 3. FXII clotting activity assays. Distribution of FXII activity in polymorphic (■) and nonpolymorphic (□) subjects of the paternal lineage; ○, unrelated controls.

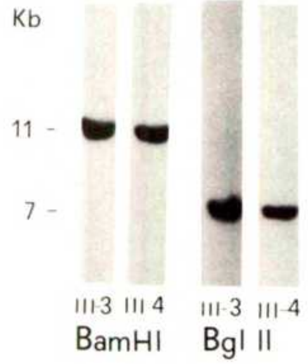

Fig 4. Exclusion of gene deletion. *Bam*HI and *Bgl*II DNA digest from parents (III-3, III-4) of the Hageman trait subjects have been hybridized to both cDNA probes.

present within 450 base pairs (bp) 3′ to the polymorphic site, and intronic sequences at least 1,200 bp long are localized 5′ to the *Taq*I additional site. The FXII gene region recognized by the 5′ FXII cDNA (350 bp) is at least 4 kb long, thus indicating that short exons are interspersed with relatively long introns. The tentative position of part of the exonic sequences is indicated in the map in Fig 5B.

DISCUSSION

A *Taq*I RFLP within the gene for FXII has been identified in two brothers carrying the Hageman trait and is also found in 11 members of their paternal lineage, thus enabling the identification of the heterozygous condition and demonstrating that the autosomal pattern of inheritance of FXII deficiency is monogenic recessive.

The presence of the polymorphism only in the paternal lineage excludes the consanguinity of parents and indicates that the two Hageman trait subjects are double heterozygous for two different FXII gene lesions. Both paternal and maternal mutations should cause the complete impairment of gene expression, as indicated by the individuals with a potential and material absence of FXII. The presence of two different lesions within the same family may suggest that this asymptomatic gene alteration could be less infrequent than suspected.

The absence of this *Taq*I intragenic polymorphism in normal unrelated individuals and its association with the reduction of FXII activity suggest that the mutation of the additional *Taq*I site could be a FXII gene lesion rather than a meaningless variant. *Taq*I restriction enzyme has been successfully used to detect other gene lesions.[11]

The *Taq*I polymorphism is localized in a portion of the FXII gene recognized by the 5′ cDNA probe. However, due to the high proportion of intronic sequences in the 5′ region, a precise assignment of exons could be obtained only if a genomic probe of known sequence were available.

This polymorphism is not produced by a gene deletion, as demonstrated by the normal patterns obtained with several restriction enzymes.

If among the possible small gene alterations a single nucleotide change is assumed, a nonsense mutation can be excluded; in fact, mutations producing additional *Taq*I recognition tetranucleotides (TCGA) in the coding sequence cannot give rise to nonsense codons. The localization of the polymorphic site in a gene region rich in intronic sequences suggests an intronic or a splice junction mutation, as is frequently found in genetic diseases.

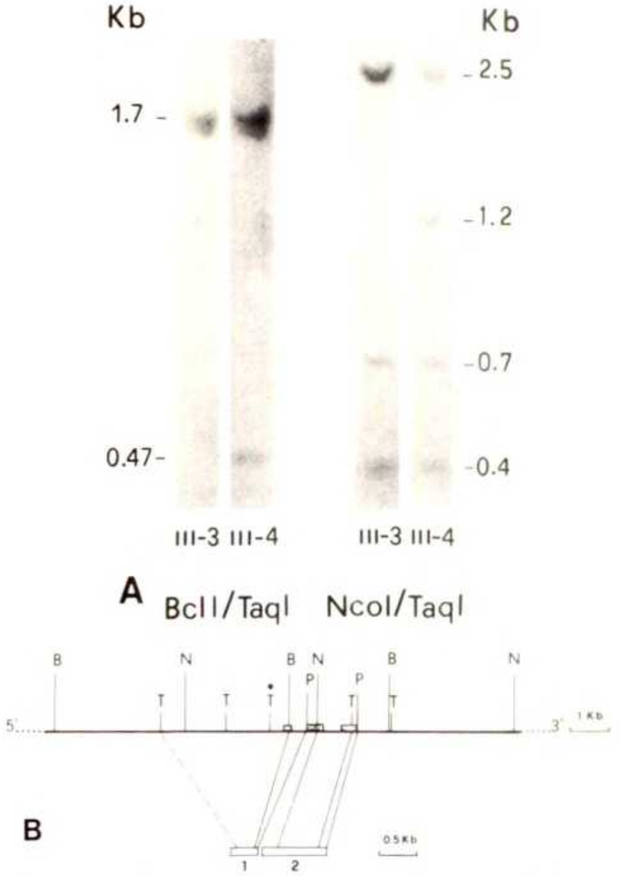

Fig 5. Localization of the *Taq*I polymorphic site in the FXII gene. (A) DNAs from a nonpolymorphic (III-3) and from a polymorphic (III-4) subject have been digested with *Bcl*I/*Taq*I and *Nco*I/*Taq*I. The hybridization has been performed with the 5′ portion of cDNA. (B) Schematic representation of restriction sites in a portion of the FXII gene. B, *Bcl*I; N, *Nco*I; P, *Pst*I; T, *Taq*I; T*, polymorphic site; 1, 5′ cDNA; 2, 3′ cDNA. Dotted boxes indicate the tentative position of part of the exonic sequences.

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Evaluation of Four Methods for Platelet Compatibility Testing

By Janice G. McFarland and Richard H. Aster

Four platelet compatibility assays were performed on serum and platelet or lymphocyte samples from 38 closely HLA-matched donor/recipient pairs involved in 55 single-donor platelet transfusions. The 22 patients studied were refractory to transfusions of pooled random-donor platelets. Of the four assays (platelet suspension immunofluorescence, PSIFT; ^{51}Cr release; microlymphocytotoxicity; and a monoclonal anti-IgG assay, MAIA), the MAIA was most predictive of platelet transfusion outcome (predictability, 74% for one-hour posttransfusion platelet recovery and 76% for 24-hour recovery). The only other assay to reach statistical significance was the PSIFT (63% predictability for one-hour posttransfusion recovery). The degree of HLA compatibility between donor and recipient (exact matches *v* those utilizing cross-reactive associations) was unrelated to the ability of the MAIA to predict tranfusion results. The MAIA may be capable of differentiating HLA antibodies, ABO antibodies, and platelet-specific antibodies responsible for failures of HLA-matched and selectively mismatched single-donor platelet transfusions.

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REFRACTORINESS to platelet transfusions continues to complicate the care of patients with hematologic malignancies and aplastic anemia. When patients respond poorly to transfusions of random-donor platelets, many can be supported with transfusions of HLA-matched, single-donor platelets.[1-4] As many as one third of the most closely HLA-matched platelet transfusions fail to achieve a satisfactory result, however.[5-9]

Platelet compatibility testing offers a potential solution to this problem. Methods used to date with varying degrees of success include indirect platelet immunofluorescence,[10,11] ^{125}I staphylococcal protein A binding,[12] enzyme-linked immunosorbent assays (ELISA),[13] and radioactive antiglobulin binding using ^{125}I-labeled polyspecific antiglobulin[14-16] or radiolabeled monoclonal anti-IgG.[10]

In the present study, we compared four assays to determine which was most useful in predicting responses to closely HLA-matched single-donor platelet transfusions of patients refractory to random-donor platelets.

MATERIALS AND METHODS

Patient selection. Twenty-two patients with bone marrow hypoplasia secondary to chemotherapy, marrow infiltration, or aplastic anemia received HLA-matched and selectively mismatched[3] platelet transfusions. All patients studied had documented poor responses to transfusions of random-donor platelet concentrates (mean one-hour posttransfusion platelet recovery, 8.5% ± 9.9% as determined by a recovery formula using an estimate of 7.5×10^{10} platelets per random-donor concentrate to calculate the random platelet dose).

Serial serum samples were collected on these patients throughout the course of platelet transfusion support and stored at −80°C until used. Transfusion events were selected for study if (1) adequate documentation was available to define the patient's clinical condition at the time of the transfusion, (2) a serum sample was obtained within 3 weeks prior to or (in several instances) shortly after platelet transfusion, and (3) the original donors were willing to return to the blood center to donate a unit or a sample of whole blood from which platelets and lymphocytes were isolated to use in the platelet compatibility tests. Patient records were examined to determine the presence or absence of fever (oral temperature greater than 38°C), sepsis, disseminated intravascular coagulation (DIC), bleeding, and the one- and 24-hour posttransfusion platelet count recovery. Transfused platelet recovery was calculated based on the following formula: Percent recovery = Body weight (kg) × 70 mL/kg × (posttransfusion platelet count − pretransfusion platelet count/μL) × 100/No. of platelets transfused × 0.67. The number of platelets transfused was calculated from the platelet count and volume of the single-donor concentrates. The factor 0.67 was used to correct for anticipated pooling of one third of the transfused cells in the spleen. One-hour posttransfusion recoveries of 65% or more and 24-hour recoveries of 57% or more were considered successful responses.

Platelet preparation. EDTA (0.6 mL, 5% EDTA/10 mL) was added to platelet-rich plasma (PRP) from units of citrate-phosphate-dextrose-A (CPD-A) whole blood. The PRP was used as a fresh platelet source, or the platelets were isolated and frozen in 5% dimethyl sulfoxide (DMSO).[17] Alternatively, 30 mL of blood in EDTA (0.3 mL, 5% EDTA/10 mL) was drawn from donors who were not eligible to donate a unit of whole blood. The PRP was separated (300 g for ten minutes), the platelets pelleted (1,000 g for seven minutes), and the platelet-poor plasma removed. The platelet button was resuspended in buffer for the ^{125}I monoclonal anti-IgG assay (MAIA) (platelet suspension buffer, PSB) or the indirect immunofluorescence test (phosphate-buffered saline with 0.1% sodium azide). Platelets were also frozen in 5% DMSO (−80°C) for use in the ^{51}Cr release assay and in the MAIA.[17]

^{125}I MAIA. Monoclonal antihuman IgG was purified from ascites fluid (BRL 9474SA, Bethesda Research Laboratories, Gaithersburg, MD) by methods previously reported.[18,19]

A method[19] adapted from that described by Lo Buglio et al[20,21] was used to quantify platelet-bound IgG. Platelets separated from EDTA whole blood or thawed in pooled AB plasma in 5% DMSO were washed three times in phosphate-buffered saline–EDTA (4 mmol/L) containing 1% bovine serum albumin (PSB) at pH 6.8 and the platelet count adjusted to 25×10^6 platelets in 0.05 mL buffer. A quantity of 0.250 mL serum (0.6 mL, 5% EDTA/10 mL) was added to the platelet suspension, and the mixture was incubated for 30 minutes at room temperature. The platelets were washed free of serum (1,000 g for seven minutes for three washes) and suspended in 0.3 mL of PSB, and 2 μg of ^{125}I-labeled monoclonal antibody in PSB (0.2 mL) was added. In preliminary studies, this amount of mono-

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clonal antibody was found to be capable of saturating IgG binding sites on platelets maximally sensitized with HLA or platelet-specific alloantibodies. After 30 minutes at room temperature, 0.1-mL aliquots containing 5×10^6 platelets were placed in triplicate in 0.4 mL Starstedt tubes over 0.2 mL 30% Percoll layers if fresh donor platelets were used. The concentration of Percoll was reduced to 20% if frozen platelets were used. The tubes were then centrifuged at 7,500 g for four minutes in a Beckman microfuge (Beckman Instruments, Inc, Fullerton, CA). The platelets migrated to the tube tips and left unbound ^{125}I-labeled monoclonal antibody above the Percoll cushion. The Percoll cushion was then carefully aspirated from the top down by using a Pasteur pipette while leaving the undisturbed platelet button at the tube tip. The tube tips were separated and placed in gamma scintillation counting tubes, and the radioactivity was determined in a gamma counter. The number of molecules of IgG bound per platelet was determined by the following formula developed by LoBuglio and colleagues[20,21]: Molecules IgG/platelet = 4.02×10^9 cpm in sample tube/specific activity $\times 5 \times 10^6$, where 4.02×10^9 is the number of molecules of monoclonal antibody/ng and specific activity is expressed as cpm per nanogram of labeled protein. The monoclonal anti-Fc antibody binds to all subclasses of IgG at a ratio of 1:1.[19,21]

For each donor platelet preparation studied, an autologous control tube using donor platelets with autologous plasma was tested in addition to a positive control using a known anti-HLA-A2 or anti-Pl^{A1} antiserum and an HLA-A2-positive, Pl^{A1}-positive platelet preparation. The mean number of molecules IgG per platelet for the autologous controls was determined, and 2 SD above the mean was chosen as the cutoff for a positive result in this assay (2,720 molecules per platelet). The mean coefficient of variation (CV) of the MAIA was 14.5% ± 6.3%.

Platelet suspension immunofluorescence test. The platelet suspension immunofluorescence test (PSIFT) used was adapted from von dem Borne et al.[22] Modifications included omission of the paraformaldehyde fixation step and reduction in the serum: cell ratio to 0.25 mL serum to 10^7 platelets. A fluorescein-conjugated polyvalent goat antihuman immune globulin was used to detect platelet-associated immunoglobulin (Meloy Laboratories, Inc, Springfield, VA). Results were graded 0 to 4+ based on the amount of fluorescence seen under the fluorescent microscope.

^{51}Chromium release test. This assay was performed on DMSO-frozen, papain-treated platelets.[17,23] Patient sera were heat inactivated and added to ^{51}Cr-labeled donor platelets. Complement in the form of normal human plasma anticoagulated with EDTA and supplemented with $MgCl_2$ was added and lysis measured by detection of the amount of released ^{51}Cr after two hours at 37°C.[23,24] Normal human serum and a known anti-Pl^{A1} antiserum were used in each assay as negative and positive controls.

Microlymphocytotoxic crossmatch. Microlymphocytotoxicity testing was done by using standard methodology[25] with patient serum and donor lymphocytes. Patient serum was used at dilutions of 1:2, 1:4, 1:8, and 1:16. Cell death was graded on a scale of 1 to 8. The scores for all dilutions were combined to arrive at a lymphocytotoxicity score. A score greater than 5 was considered a positive result.

Statistical analysis. Two-by-two chi-square comparisons of antibody test results with one- and 24-hour posttransfusion platelet recoveries were performed for all tests by using the cutoff values for successful transfusion responses defined before.

For purposes of this study, the following definitions of sensitivity, specificity, and predictability were used: Sensitivity = true positives/(true positives + false negatives). Specificity = true negatives/(true negatives + false positives). Predictability = (true positives + true negatives)/all test results. True positive refers to those donor-recipient pairs with positive antibody test results and poor transfusion responses, true negative to negative test results with satisfactory transfusion responses, false-positive to positive test results and satisfactory responses, and false-negative to negative test results and poor transfusion responses.

RESULTS

In all, 55 transfusions were studied. Thirty-five transfusions from 25 donors administered to 17 patients were documented to occur in the absence of fever, bleeding, splenomegaly, sepsis, or DIC (group 1). One-hour posttransfusion platelet recoveries were available on all 35 transfusions in group 1; 24-hour recoveries were available in 34. Twenty transfusions from 17 donors were given to 14 patients in whom one or more of the clinical factors listed before were present (group 2). One-hour recovery data were available on all 20 of the group 2 patients, and in 19, 24-hour data were available. Nine patients received transfusions classified in groups 1 and 2 because of changes in their clinical status. Serum samples from the patients were collected a mean of 3.5 ± 8.1 days prior to transfusion. Forty-six samples were collected pretransfusion (zero to 22 days), and nine were collected posttransfusion (one to 15 days).

Results of all four assays were correlated with the transfusion responses for group 1 transfusions. Sensitivity, specificity, and predictability at one and 24 hours were calculated for each assay (Table 1). Of the four assays, the MAIA was most predictive of transfusion outcome (Fig 1). At 24 hours, its specificity was 100%, ie, no false-positive reactions were obtained. The only other assay to reach statistical significance in predicting recovery was the PSIFT. However, PSIFT had only 40% sensitivity at one hour. When group 1 and group 2 transfusions were combined, the ability of the MAIA test to predict transfusion responses at one and 24 hours was reduced (Fig 2). Even so, specificity at 24 hours was 86%.

Donor-recipient matches utilizing cross-reactive HLA associations (B1X, B2X, B3X, B2UX) (Table 2)[4] were compared with those that were more completely matched (A, B1U, B2U). At one hour, the response to the cross-reactive matches was predicted less well by the MAIA (Fig 3A) than

Table 1. Prediction of Transfusion Responses

Assay	1-h Recovery		24-h Recovery
MAIA	$\chi^2 = 8.11, P < .01$		$\chi^2 = 11.77, P < .001$
	Sensitivity	75%	69%
	Specificity	73%	100%
	Predictability	74%	76%
LCT	χ^2-NS		NS
	Sensitivity	24%	30%
	Specificity	73%	88%
	Predictability	47%	45%
^{51}Cr release	χ^2-NS		NS
	Sensitivity	12%	10%
	Specificity	100%	100%
	Predictability	48%	32%
PSIFT	$\chi^2 = 4.97, P < .05$		NS
	Sensitivity	40%	31%
	Specificity	93%	88%
	Predictability	63%	44%

Abbreviations: LCT, lymphocytotoxicity test; NS, not significant.

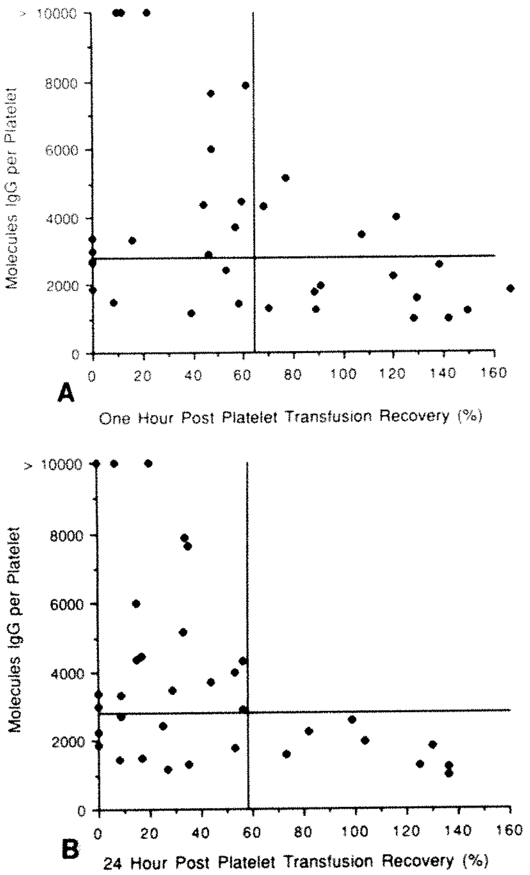

Fig 1. Crossmatch results using the MAIA. One-hour (A) and 24-hour (B) platelet recoveries (abscissa) and number of molecules of IgG measured per platelet in the MAIA crossmatch (ordinate) are shown for group 1 transfusions. A total of 2,720 molecules per platelet or greater was considered a positive result based on the mean number of molecules per platelet plus 2 SD in the normal autologous controls (n = 34). A successful one-hour recovery was considered to be 65% and a successful 24-hour recovery, 57%. $\chi^2 = 8.11$, $P < .01$ for one hour recovery; $\chi^2 = 11.77$, $P < .001$ for 24-hour recovery (2 × 2 chi-squared test of homogeneity).

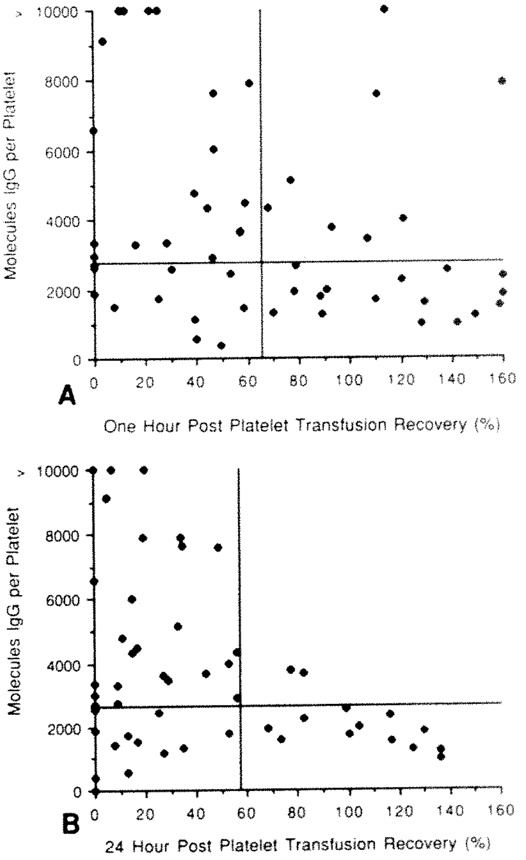

Fig 2. Crossmatch results using the MAIA for all transfusions in groups 1 and 2 at one hour (A) and 24 hours (B). Units are as in Fig 1. $\chi^2 = 6.24$, $P < .05$ for one-hour recovery, $\chi^2 = 10.25$, $P < .01$ for 24-hour recovery (2 × 2 chi-squared test of homogeneity).

the response to fully matched transfusions (Fig 3B). This difference was less apparent at 24 hours (Fig 4A and 4B).

When the mean of the positive reactions for the cross-reactive group of transfusions was compared with that of the fully matched group at 24 hours, we found that positive reactions in the latter group were characterized by levels of platelet-associated IgG about twice as great (7,310 molecules per platelet) as those of the former (3,174 molecules per platelet) (Fig 5).

One patient's serum (G.M.) gave the highest reactions of any donor-recipient pair when tested against platelets of her donor (66,683 molecules per platelet) (Fig 5). These HLA-A–matched, ABO-matched platelets gave poor one- and 24-hour platelet count recoveries (10% and 7%).

ABO antibodies have been implicated as a cause of refractory platelet responses in some patients. The ABO blood groups of both recipients and donors were analyzed for cross-reactive and fully matched transfusions. In evaluating the 24-hour data, four of nine positive reactions noted in the fully matched group were found to be associated with transfusion of platelets from an A or AB donor to a group O patient. Only one of the positive reactions in the cross-reactive group represented this type of incompatibility (Fig 4). These numbers were too small to allow meaningful statistical analysis.

One group O patient with a previously demonstrated high-titer anti-A in his serum (agglutinating and hemolytic) was included in the study. Three transfusions from group A donors (HLA match grades A, B2U, B1X) and two transfusions from group O donors (HLA match grade B2UX) were

Table 2. Classification of Donor/Recipient Pairs on the Basis of HLA Match

A	All 4 antigens in donor identical to those of recipient
B1U	Only 3 antigens detected in donor, all present in recipient.
B2U	Only 2 antigens detected in donor, both present in recipient.
B1X	Three donor antigens identical to recipient, fourth antigen cross-reactive with recipient.
B2UX	Only 3 antigens detected in donor: 2 identical with recipient, third cross-reactive.
B2X	Two donor antigens identical to recipient, third and fourth antigens cross-reactive with recipient.

From Duquesnoy et al.[3]

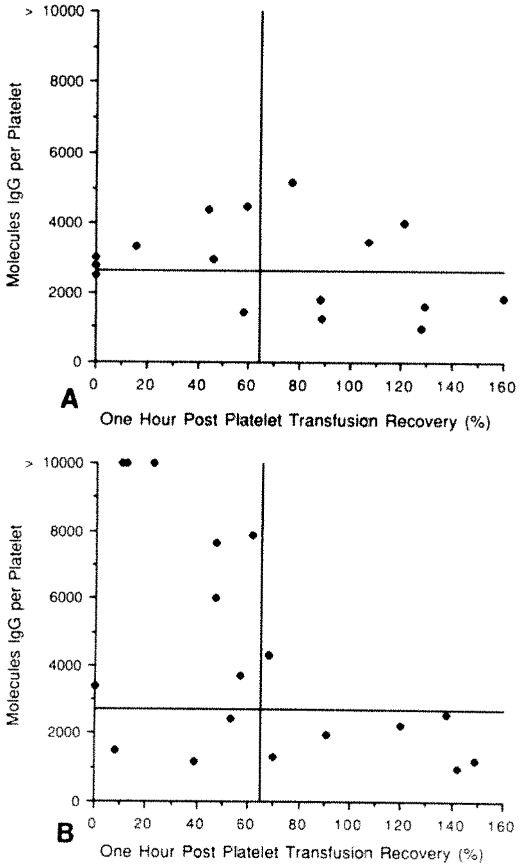

Fig 3. Crossmatch results using the MAIA and one-hour platelet recoveries for group 1 transfusions in which donor-recipient pairs were matched only within cross-reactive HLA antigen groups (A) or were more closely matched (B). Units are as in Fig 1. χ^2 not significant for transfusions in (A); $\chi^2 = 5.83$, $P < .05$ for transfusions in (B) (2 ×2 chi-squared test of homogeneity).

analyzed. The mean MAIA result for group A transfusions was 9,595 ± 1,473 molecules IgG per platelet (Fig 5), and that of the group O platelets was 689 ± 420 molecules IgG per platelet. These MAIA results correlated with the transfusion responses: group A transfusions, 12%, 19%, 0% recovery at one hour; and group O transfusions, 128%, 49% recovery at one hour.

DISCUSSION

Of the four methods studied, the MAIA test was most predictive of transfusion response at one and 24 hours. The majority of the MAIA assays were performed on frozen donor cells, which suggests that frozen platelets can be used to select donors for refractory patients. In contrast, the PSIFT lacked sensitivity in our hands. Others have found this assay to be more sensitive.[11] Two possible explanations are (1) the lower serum:cell ratio used in our studies and (2) the fact that all transfusions studied by us were matched or only slightly mismatched for class I HLA antigens. It may be that more extreme HLA incompatibilities are necessary to demonstrate positive reactions in the PSIFT. The lymphocytotoxicity test was not predictive of the transfusion response. This is probably due to the fact that closely HLA-matched donors were used for these patients. Antiglobulin enhancement of the microcytotoxicity test, not used in this study, might have improved the sensitivity of the lymphocyte crossmatch.[26] The failure of the ^{51}Cr release method to predict platelet transfusion responses probably reflects the requirement of this assay for complement-fixing antibodies.

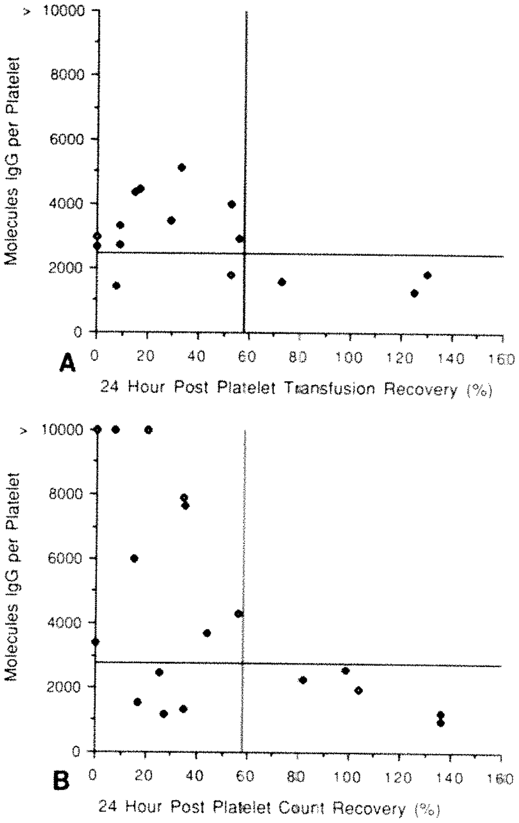

Fig 4. Crossmatch results using the MAIA and 24-hour platelet recoveries for group 1 transfusions in which donor-recipient pairs were matched within cross-reactive HLA antigen groups (A) or were more closely matched (B). ABO-incompatible transfusions are shown by open symbols. $\chi^2 = 5.62$, $P < .05$ for (A); $\chi^2 = 6.92$, $P < .01$ for (B) (2 × 2 chi-squared test for homogeneity).

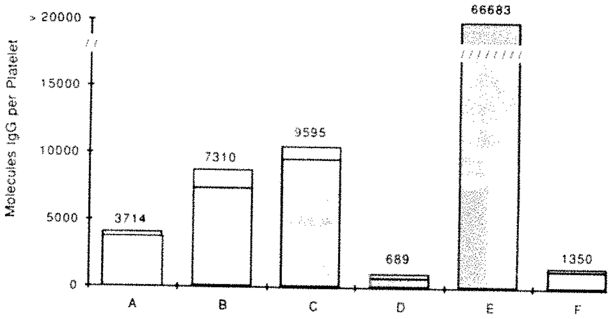

Fig 5. Crossmatch results (mean + SEM) for positive reactions obtained with donor-recipient pairs matched only within HLA cross-reactive antigen groups (A); more closely matched (B); mismatched for ABO but matched for HLA, Group O patient with high-titer anti-A (n = 3) (C); matched for ABO and HLA and transfused to the same patient (n = 2) (D); matched for ABO and HLA and tested against serum from patient G.M. (E); and control studies using autologous serum (F).

Although the MAIA provided the best prediction of transfusion response, occasional false-positive and, more common, false-negative reactions were noted, particularly when recoveries one hour after the transfusion were evaluated. The false-positive reactions for one-hour recovery predictions may reflect the high sensitivity of the MAIA for detection of platelet-bound IgG, ie, it may be capable of detecting amounts of IgG insufficient to cause poor one-hour recoveries but sufficient to cause platelet destruction in 24 hours. False-negative reactions might be explained by the inability of our monoclonal antibody to detect immunoglobulins other than IgG. Alternatively, nonimmune factors causing platelet consumption that were not apparent clinically may have affected recoveries in some patients.

It is evident that the MAIA predicted responses to transfusions that were apparently matched for all class I HLA-A and -B antigens as well as those that were slightly mismatched (cross-reactive) (Fig 4). The positive crossmatches seen with platelets of match grades A and BU could be caused by antibodies reactive with platelet-specific antigens or with A or B blood group substances. Subtle Class I HLA antigen differences not characterized by available serological reagents might also explain the positive reactions seen with these well-matched but unrelated donor/recipient pairs. The larger number of ABO-incompatible transfusions in the fully matched group with positive crossmatches supports the possibility that ABO antibodies are important in some of these positive reactions. Deposition of extremely high levels of IgG by one patient's serum (G.M.) onto donor cells (Fig 5) suggests the presence of one or more antibodies reacting with platelet-specific antigens that are expressed more densely on the platelet surface than HLA antigens.[19]

These preliminary results showing differences in the frequency of positive crossmatches and the strength of individual reactions with HLA-cross-reactive, HLA-fully matched, and ABO-incompatible donor-recipient pairs suggest that the MAIA may be useful in distinguishing HLA from non-HLA seroreactivity as the cause of platelet refractoriness in alloimmunized patients. Larger numbers of transfusions must be analyzed to investigate this possibility.

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Kinetics of Inhibition of Human Plasma Kallikrein by a Site-Specific Modified Inhibitor Arg^{15}-Aprotinin: Evaluation Using a Microplate System and Comparison With Other Proteases

By Cheryl F. Scott, Herbert R. Wenzel, Harald R. Tschesche, and Robert W. Colman

Human plasma kallikrein, a product of contact-activated plasma proteolysis, is moderately inhibited by aprotinin, a small polypeptide from bovine lung that has been used as an experimental drug in human disease states. Aprotinin has a Lys residue in the P1 (reactive center) position occupying residue 15. Since kallikrein is an arginine-directed serine protease, we hypothesized that an altered form of aprotinin, Arg^{15}-aprotinin, might be a better inhibitor. Kinetic evaluations were performed in 96-well microplates. We found that the K_L (loose or Michaelis-Menten complex) was unchanged by the modification. However, the association rate constant was increased from 1.14×10^4 (mol/L)$^{-1}$s^{-1} to 1.5×10^5 (mol/L)$^{-1}$s^{1}, thus indicating that the inhibition rate was increased 14-fold for the modified protein. The K_i (at equilibrium) was decreased from 3.2×10^{-7} mol/L to 1.5×10^{-8} mol/L after substituting Arg for Lys in the P1 position. Therefore, the modified inhibitor binds to plasma kallikrein more tightly than the natural protein. We also investigated the effect of Arg^{15}-aprotinin on tissue kallikrein, plasmin, factor XIIa, factor XIa, and thrombin and found that the K_i slightly decreased from 5.1×10^{-7} mol/L to 1.2×10^{-7} mol/L for tissue kallikrein and slightly decreased from 2×10^{-8} mol/L to 1×10^{-8} mol/L for plasmin. Arg^{15}-aprotinin did not inhibit thrombin or factor XIIa, even though both enzymes are arginine-directed serine proteases. However, factor XIa, although it was not inhibited by aprotinin, had a K_i of 3.4×10^{-8} mol/L for Arg^{15}-aprotinin. Therefore, Arg^{15}-aprotinin is a more effective inhibitor of plasma kallikrein as well as factor XIa but shows minimal preference for plasmin and tissue kallikrein. This study also indicates that it is possible and practical to perform kinetic analyses directly in microplates.

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HUMAN PLASMA PREKALLIKREIN is one of three proenzymes constituting the contact system of plasma proteolysis. Activation of this system, which includes the conversion of prekallikrein to kallikrein in both the surface and fluid phases, has been implicated in the hypotension that accompanies septicemia[1] and in attacks of hereditary angioedema.[2-4] Plasma kallikrein has been shown, in vitro, to be moderately inhibited by aprotinin,[5] a potent inhibitor of trypsin and plasmin.

Aprotinin, a polypeptide with a molecular weight of 6,512 d, contains a lysyl residue at position 15 that functions as the P1 or reactive center of the inhibitor.[6,7] Thus, the Lys^{15}-Ala^{16} bond, located at the top of the molecule, specifically interacts with the active site of the enzyme.[8] However, this reaction is stabilized by additional weaker interactions,[9,10] and the most intimate contacts with the enzyme are made by the residues in positions 15, 16, 17, and 39.[10] Inhibition of serine proteases by aprotinin has always been reported to be reversible and competitive.[5]

Plasma kallikrein is a serine protease with a preference for arginyl residues in the P1 position.[11,12] We therefore postulated that if arginine were substituted for lysine in the P1 position of aprotinin a more potent inhibitor for plasma kallikrein might result that could have potential clinical usefulness. Such a modified aprotinin was recently synthesized enzymatically with arginine substituted for lysine at amino acid 15 (Arg^{15}-aprotinin), the reactive center of the aprotinin molecule.[13]

Traditionally, characterization of enzyme functions has been performed with a spectrophotometer using initial rate determinations or, in some cases, measuring the change in absorbance of a chromophore after a fixed time of substrate hydrolysis. In this report, we investigated in detail the kinetics of inhibition by Arg^{15}-aprotinin on human plasma kallikrein by using the chromogenic substrate H-D-Pro-Phe-Arg-pNA (S-2302), 96-well microplates, and a microplate reader. We then compared the effect of the two inhibitors on five other human enzymes, urinary kallikrein, plasmin, factor XIIa, factor XIa, and thrombin.

MATERIALS AND METHODS

Human plasma kallikrein, plasmin, H-D-Pro-Phe-Arg-pNA (S-2302), H-D-Val-Leu-Lys-pNA (S-2251), H-D-Phe-Pip-Arg-pNA (S-2238), and H-D-Val-Leu-Arg-pNA (S-2266) were generously furnished by KabiVitrum, Mölndal, Sweden, and its US distributor, Helena Laboratories, Beaumont, TX. Human thrombin (2,900 U/mg) was a generous gift of Dr John W. Fenton II, Albany, NY. Microplates utilized in this study were Falcon, tissue culture–treated plates (#3075) purchased from Fisher Scientific Company, King of Prussia, PA. The microplate reader was a Bio-Rad model 2550 EIA reader purchased from Bio-Rad, Inc, Richmond, CA. Polyethylene glycol (PEG 8000) was purchased from Sigma Chemical Co, St Louis. All other reagents were the best grade available.

Human plasma kallikrein. Obtained from KabiVitrum, human plasma kallikrein is purified 200-fold from euglobulin-free plasma and chromatographed on QAE-Sephadex, Sephadex G-150, and Sephadex G-100 according to the method of Gallimore et al.[14] The specific activity is 1.2 Chromozyme-PK U/mg and contains IgG as the only protein contaminant.

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Human urinary kallikrein. A generous gift of Dr Julie Chao (Charleston, SC), human urinary kallekrein was purified to homogeneity according to the method of Shimamoto et al.[15] The specific activity was 74 EU/mg using the substrate Tos-Arg-O-[^{3}H]-Me.

Human plasmin. Human plasmin (KabiVitrum) is generated from plasminogen that is prepared according to the method of Deutsch and Mertz[16] and activated via a matrix-bound activator. The specific activity is 15 casein units (CU)/mg.

Human factor XIa. It was purified and activated as described previously by this laboratory.[17] The specific activity was 200 U/mg where 1 unit is the amount in 1 mL normal plasma.

Human factor XIIa. Human factor XIIa was prepared as previously detailed from our laboratory.[18] The specific activity was 62 U/mg where 1 unit is the amount in 1 mL normal plasma.

Aprotinin. Aprotinin was purified from bovine lung as described by Fritz et al.[19]

Arg^{15}-aprotinin (semisynthetic). The procedure for replacement of Lys by Arg in position 15 of aprotinin is described in detail elsewhere.[20] Briefly, it consists of the following steps: (1) selective reduction of the Cys^{14}-Cys^{38} disulfide bridge with sodium borohydride[21]; (2) tryptic cleavage of the reactive-site peptide bond Lys^{15}-Ala^{16}[22]; (3) air reoxidation of the half-cystine residues to yield modified aprotinin[22]; (4) removal of Lys^{15} by carboxypeptidase B[20]; (5) coupling of arginine to Cys^{14} in a reverse reaction catalyzed by carboxypeptidase B to yield modified Arg^{15}-aprotinin in minute amounts[20]; (6) trapping of this product by complex formation with kallikrein from porcine pancreas, thus shifting the carboxypeptidase-catalyzed reaction towards synthesis; and (7) kinetic control dissociation of the complex to yield single-chain Arg^{15}-aprotinin as the main product.

The semisynthetic inhibitor was purified by gel filtration and cation-exchange chromatography. It was characterized by amino acid analysis, automated Edman degradation, x-ray crystal structure analysis,[23] inhibition studies with various serine proteinases, polyacrylamide gel electrophoresis, and high-pressure liquid cation-exchange chromatography.[13] The Arg^{15}-aprotinin preparation was found to be homogeneous according to all these criteria applied.

Microassay for plasma kallikrein. Plasma kallikrein was reconstituted with 0.2% PEG to prevent nonspecific adsorption of proteases.[24] The total volume of enzyme plus buffer and/or inhibitor was routinely 100 μL. Fifty microliters of 2 mmol/L S-2302 was added to the enzyme mixture at room temperature, and hydrolysis was allowed to proceed for 15 to 20 minutes. The hydrolysis reaction was quenched with 50 μL of 50% acetic acid. The microplates were read at 405 nm, and the blanks (omitting enzyme) were subtracted from the total absorbance, or the values were plotted directly with the "blank value" at the origin. The amount of paranitroanaline (pNA) produced was calculated from a standard curve prepared from 200 μL of various concentrations of pNA standard (available free of charge from KabiVitrum/Helena Laboratories), or the absolute absorbance values at 405 nm were used. The buffer used for this assay was 20 mmol/L Tris-Cl, pH 7.4, containing 0.15 mol/L NaCl, 1 mmol/L EDTA, and 0.1% PEG (buffer A).

Determination of K_m for plasma kallikrein and S-2302 in a microplate system. In preliminary experiments, the rate of hydrolysis of S-2302 was linear over 30 minutes in the range of substrate concentrations used, which allowed a single time of 20 minutes to be used for estimation of the initial rates. In addition, the degree of hydrolysis at a single time and substrate concentration was proportional to the kallikrein concentration in the presence or absence of inhibitor (Fig 1A), even at a low substrate concentration (0.13 mmol/L).

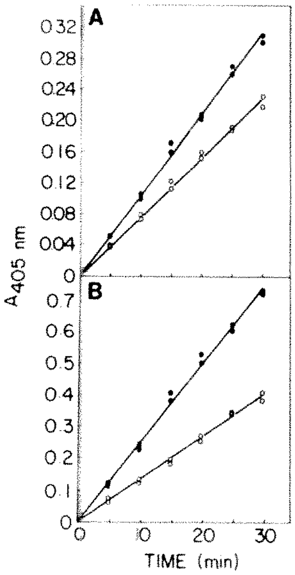

Fig 1. Progress curves of plasma kallikrein in the presence and absence of aprotinin. (A) Kallikrein (3 nmol/L) was incubated with 0.133 mmol/L S-2302 in the presence (○) and absence (●) of 0.25 μmol/L aprotinin. At the times indicated, the reaction was quenched with acetic acid (see Materials and Methods). (B) Kallikrein (3 nmol/L) was preincubated with 0.5 μmol/L aprotinin (○) or buffer (●) for 30 minutes prior to the addition of 0.66 mmol/L S-2302. At the times indicated, the reaction was quenched with acetic acid (see Materials and Methods).

When kallikrein was preincubated with aprotinin for 30 minutes prior to the addition of S-2302, linear progress curves resulted (Fig 1B). Significant dissociation did not occur due to minimal dilution of enzyme and prevention of dissociation by the substrate.

When kallikrein was incubated with S-2302 ranging from 0.13 to 0.66 mmol/L (conditions as in Materials and Methods), the K_m was found to be 0.25 mmol/L, in close agreement with previously published results using S-2302 in a spectrophotometric assay.[25] Therefore, after interpreting the results of these experiments, we proceeded to use the microsystem for further kinetic analyses.

Kinetic analysis of inhibition. The equation used was adapted from that of Fritz and Wunderer[5] for a reversible tight binding inhibitor:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C$$

where E = kallikrein, I = aprotinin or Arg^{15}-aprotinin, L = loose or Michaelis-Menten complex, and C = stable complex. $K_L = k_{-1}/k_1$; $K_i = k_{-2}/k_2$.

Since the formation of L is a fast second-order reaction, k_2 is assumed to be rate determining for the formation of a stable complex and is approximately equal to the k_{assoc} for the complex.

Plasma kallikrein—determination of K_i. Kallikrein was incubated with various concentrations of aprotinin or Arg^{15}-aprotinin for 30 minutes prior to the addition of S-2302. The hydrolysis of the substrate by the residual enzyme activity proceeded for 20 minutes before termination by acetic acid.

Human thrombin—determination of K_i. Twenty microliters of human thrombin (80 nmol/L) was incubated with either aprotinin, Arg^{15}-aprotinin, or buffer A in a final volume of 120 μL for 30 minutes prior to the addition of 60 μL of 0.75 mmol/L S-2238. Hydrolysis proceeded for three minutes prior to the addition of 50 μL of 50% acetic acid.

Human plasmin—determination of K_i. Fifty microliters of human plasmin (0.125 CU/mL) was incubated with either aprotinin, Arg^{15}-aprotinin, or buffer (50 mmol/L Tris-Cl, pH 7.4, containing 0.1% PEG) in microplates (final volume, 100 μL) for 30 minutes prior to the addition of 50 μL of 2 mmol/L S-2251. Hydrolysis proceeded for 20 minutes prior to the addition of 50 μL of 50% acetic acid.

Human urinary kallikrein—determination of K_i. Fifty microliters of human urinary kallikrein (10 μg/mL) was incubated with aprotinin, Arg^{15}-aprotinin, or buffer A in a final volume of 100 μL for 30 minutes prior to the addition of 50 μL of 0.8 mmol/L S-2266. Hydrolysis proceeded for 20 minutes prior to the addition of 50 μL of 50% acetic acid.

Human factor XIa—determination of K_i. Fifty microliters of human factor XIa (0.04 U/mL) was incubated with either aprotinin, Arg^{15}-aprotinin, or buffer A in a final volume of 100 μL for 30 minutes. Fifty microliters of S-2266 (6 mmol/L) was added, and hydrolysis proceeded for ten minutes prior to quenching with 50 μL of 50% acetic acid.

Human factor XIIa—determination of K_i. Fifty microliters of human factor XIIa (0.02 U/mL) was incubated with either aprotinin, Arg^{15}-aprotinin, or buffer A in a final volume of 100 μL for 30 minutes. Fifty microliters of S-2302 (2 mmol/L) was added, and hydrolysis proceeded for 90 minutes prior to the addition of 50 μL of 50% acetic acid.

RESULTS

Determination of K_L of plasma kallikrein with aprotinin and Arg^{15}-aprotinin. Kallikrein was added to various concentrations of either aprotinin (Fig 2, left) or Arg^{15}-aprotinin (Fig 2, right) together with S-2302 and was assayed as in Materials and Methods. Inhibition was competitive and reversible for both inhibitors, similar to the action of aprotinin on other enzymes.[26] From the data in Fig 2, the K_m and the K_m (app) were plotted *v* inhibitor concentration to determine the K_L (loose or Michaelis complex), which was calculated to be 1.02×10^{-7} mol/L, for both inhibitors (Fig 3).

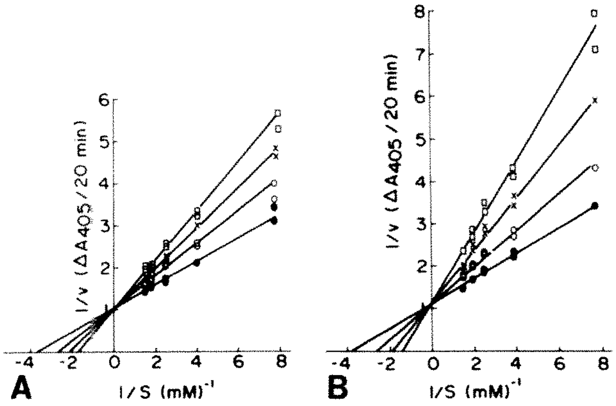

Fig 2. Determination of K_m and K_m app for plasma kallikrein with aprotinin and Arg^{15}-aprotinin. Kallikrein (3 nmol/L) was incubated with aprotinin (A) or Arg^{15}-aprotinin (B) ranging from 64 to 192 nmol/L in the presence of S-2302 ranging from 0.133 to 0.66 mmol/L. The hydrolysis of the substrate proceeded for 20 minutes before quenching with acetic acid. ●, 0; ○, 64 nmol/L aprotinin or Arg^{15}-aprotinin; ×, 128 nmol/L aprotinin or Arg^{15}-aprotinin; □, 192 nmol/L aprotinin or Arg^{15}-aprotinin.

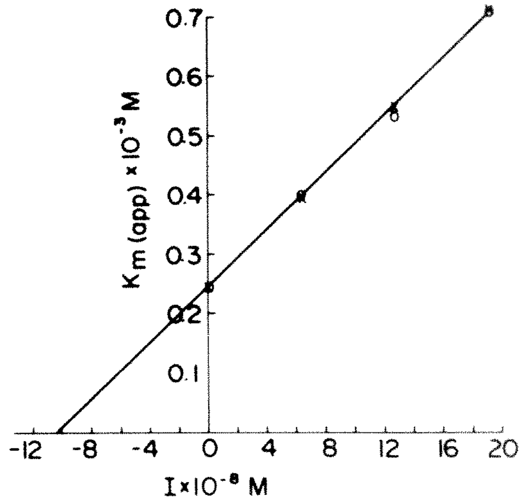

Fig 3. Calculation of K_L for plasma kallikrein with aprotinin and Arg^{15}-aprotinin. The K_m and K_m (app) from Fig 2 were plotted *v* the inhibitor concentration. The intercept on the abscissa is equal to the K_L (loose, Michaelis-Menten inhibition constant). ×, aprotinin; O, Arg^{15}-aprotinin.

Determination of k_2 (k_{assoc}) of the complex of plasma kallikrein with aprotinin and Arg^{15}-aprotinin. Kallikrein was incubated with an equimolar concentration of Arg^{15}-aprotinin (Fig 4) for various times prior to addition of S-2302. The association rate constant (k_2) for Arg^{15}-aprotinin was found to be 1.5×10^5 (mol/L)$^{-1}$s^{-1}.

Kallikrein (20 nmol/L) was incubated with aprotinin ranging in concentration from 150 nmol/L to 600 nmol/L. At various times, a portion was transferred to a microplate containing S-2302, and the hydrolysis proceeded as in Materials and Methods. By either a first-order plot or calculation by the second-order rate equation, the k_{assoc} was calculated to be 1.14×10^4 (mol/L)$^{-1}$ s^{-1} (data not shown). Therefore, the

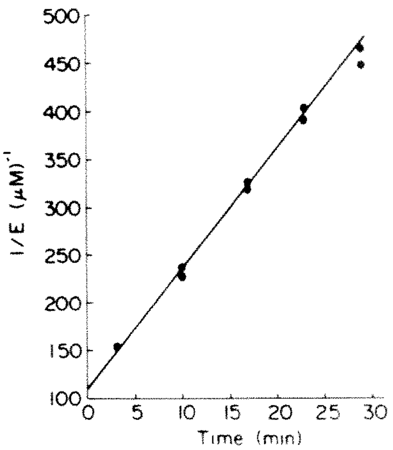

Fig 4. Determination of association rate constant (k_2) of plasma kallikrein and Arg^{15}-aprotinin. Kallikrein (9 nmol/L) was incubated with Arg^{15}-aprotinin (9 nmol/L). At various times, a portion was assayed for residual kallikrein activity. The reciprocal of the residual enzyme activity is plotted *v* time. The slope is equal to the k_2.

association rate for kallikrein and the modified inhibitor is ninefold greater than that for the natural inhibitor.

Determination of the equilibrium constant (K_i) of plasma kallikrein and aprotinin or Arg^{15}-aprotinin. The K_i for kallikrein and aprotinin was calculated to be 3.2×10^{-7} mol/L (Fig 5A) and 1.5×10^{-8} mol/L for kallikrein and Arg^{15}-aprotinin (Fig 5B). Therefore, the affinity of Arg^{15}-aprotinin for plasma kallikrein is 20-fold tighter than for the unmodified inhibitor.

Calculation of the k_{-2} for kallikrein and aprotinin or Arg^{15}-aprotinin. The k_{-2} was calculated by the following equation:

$$k_{-2} = K_i(k_2).$$

The k_{-2} for kallikrein and aprotinin was calculated to be $3.6 \times 10^{-3} s^{-1}$ and $2.5 \times 10^{-3}/s$ for kallikrein and Arg^{15}-aprotinin. The half-life of the complex ($t^{1/2} = 0.693/k_{-2}$) would therefore be 3.2 minutes for kallikrein inhibited by aprotinin and 4.6 minutes for kallikrein inhibited by Arg^{15}-aprotinin.

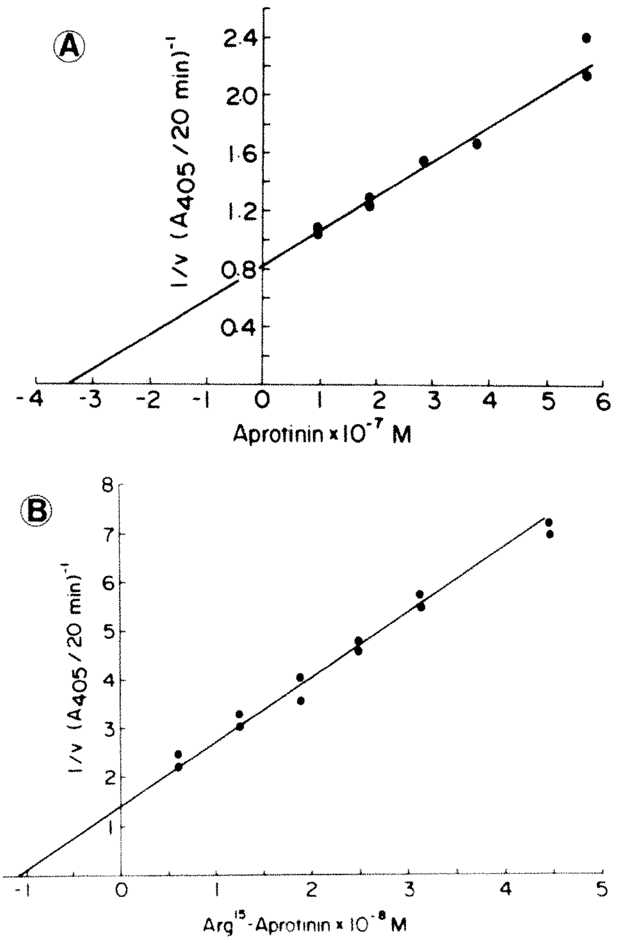

Fig 5. (A) Determination of K_i of plasma kallikrein and aprotinin. Kallikrein (9 nmol/L) was preincubated with aprotinin ranging from 95 to 570 nmol/L for 30 minutes prior to the incubation with S-2302 for 20 minutes. The reciprocal of the A_{405}/20 minutes is plotted against the aprotinin concentration. The intercept on the abscissa is equal to the dissociation constant. (B) Determination of K_i of plasma kallikrein and Arg^{15}-aprotinin. Kallikrein (9 nmol/L) was preincubated with Arg^{15}-aprotinin ranging from 6.4 to 44.8 nmol/L for 30 minutes prior to the incubation with S-2302 for 20 minutes. The data were treated as per Fig 5A.

Table 1. Equilibrium Dissociation Constant (K_i) for Proteases and Aprotinin or Arg^{15}-Aprotinin at 23°C (pH 7.4)

	Aprotinin	Arg^{15}-Aprotinin
Plasma kallikrein	3.2×10^{-7}mol/L	1.5×10^{-8}mol/L
Tissue kallikrein*	5.1×10^{-7}mol/L	1.2×10^{-7}mol/L
Plasmin	2×10^{-8}mol/L	1×10^{-8}mol/L
Thrombin	†	†
Factor XIIa	†	†
Factor XIa	†	3.4×10^{-8}mol/L

The details of the incubation mixtures and assay conditions are in Materials and Methods. Kinetic analyses are performed as in Fig 5.

*Human urinary kallikrein.

†No inhibition.

Comparison of inhibitor potency for aprotinin and Arg^{15}-aprotinin on other proteases. The evaluation of the interaction of aprotinin and Arg^{15}-aprotinin on human urinary kallikrein, human plasmin, human factor XIIa, human factor XIa, and human thrombin is presented in Table 1. Arg^{15}-aprotinin was a much more potent inhibitor than aprotinin for plasma kallikrein, but only slightly more potent for plasmin and tissue kallikrein. Thrombin and factor XIIa were not inhibited by either aprotinin or by Arg^{15}-aprotinin. Although factor XIa was not inhibited by aprotinin, it was potently inhibited by the modified inhibitor.

DISCUSSION

The clinical use of aprotinin has been described for pancreatitis[27-30] and other pathophysiological conditions with varying degrees of success. Since the K_i value for the kallikrein-aprotinin complex (3.2×10^{-7} mol/L) approaches the in vivo concentration of plasma prekallikrein (4.5×10^{-7} mol/L), a considerable molar excess of aprotinin would be needed to inhibit any liberated kallikrein.[5] This study indicates that Arg^{15}-aprotinin is a more efficient inhibitor of kallikrein than aprotinin since there is a 13-fold increase in the rate of association and a 20-fold decrease in the dissociation rate of the complex. These advantages make it more attractive for the clinical inactivation of plasma kallikrein since the rate of inactivation is sixfold greater than that obtainable with plasma concentrations of $C1^-$ inhibitor, the major plasma protease inhibitor of plasma kallikrein.[31] However, the reversible nature of the inhibitor may limit its application to acute situations since, with a half-life of the tight enzyme-inhibitor complex equal to 4.6 minutes, continuous infusions of relatively high concentrations would have to be used to produce the tight binding necessary to obviate the presence of low concentrations of this pathologically produced biologically potent enzyme because dilution in the plasma would favor dissociation.

The previous determinations of the K_i of aprotinin for human plasma and tissue kallikreins were performed at pH 8.0.[5] Since Engel et al[32] demonstrated that both the k_2 and the K_i are pH dependent, we chose to perform all kinetic analyses under similar conditions of pH and ionic strength in order to achieve a more accurate comparison of the binding of both aprotinin and Arg^{15}-aprotinin to various plasma proteases. Furthermore, the pH and ionic strength of our buffers closely approximates the conditions in blood. Under these conditions, plasma kallikrein and tissue kallikrein have similar affinities for aprotinin, and the K_i is further lowered for plasma kallikrein by substitution of the arginine residue in position 15. Plasmin, which appears to prefer lysyl residues, was minimally affected by the substitution, similar to the lack of effect of arginine in the P1 position of the homologous sea anemone inhibitor.[33] The most striking change, however, was with factor XIa where aprotinin failed to inhibit this enzyme, whereas Arg^{15}-aprotinin had a very high affinity. This finding is similar to the marked increase in factor XIa inhibition seen with an arginine substitution in the P1 position of α_1-antitrypsin.[34] Therefore, Arg^{15}-aprotinin is an efficient inhibitor of factor XIa, an enzyme for which few inhibitors have been described.

The P1 position of an inhibitor molecule has been postulated to be the most critical determinant of inhibitory specificity.[11] However, if the P1 residue were the only crucial determinant of inhibitor function, then Arg^{15}-aprotinin should have been an excellent inhibitor of thrombin and factor XIIa since both are arginine-directed serine proteases. Since Arg^{15}-aprotinin did not inhibit either enzyme, it appears that the P2 as well as other residues are also important contributors to inhibitory function.

The use of the microplate system for formal kinetic analyses can drastically reduce assay time as well as substantially cut the cost of each determination. The system also obviates the need for a thermostated, sophisticated spectrophotometer. Furthermore, microplate assays can be performed virtually anywhere since the only requirement is a small bench area. This new technology should prove useful in any situation where a chromogenic substrate is used.

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Expression of the *c-raf* Protooncogene in Human Hematopoietic Cells and Cell Lines

By Eric Sariban, Thomas Mitchell, and Donald Kufe

The murine sarcoma virus 3611 contains the transforming *v-raf* gene that has partial nucleotide homology with the *src* family of tyrosine kinase-encoding oncogenes. Although this virus induces fibrosarcomas in mice, a recombinant murine retrovirus carrying both the *raf* and *myc* oncogenes induces immunoblastic lymphomas and immortalizes mouse macrophages in vitro. The present study has thus monitored the expression of *c-raf* in human hematopoietic cells. The results demonstrate the presence of a 3.6-kb *c-raf* transcript in HL-60 promyelocytic leukemic cells. The induction of HL-60 cell differentiation along the monocytic or granulocytic lineages had no detectable effect on the level of *c-raf* transcripts. Furthermore, in contrast to *c-myc* and *c-fms* expression, inhibition of protein synthesis with cycloheximide had no detectable effect on *c-raf* expression. Similar levels of *c-raf* RNA were also found in other human cell lines derived from myeloid, B cell, and T cell tumors, as well as in normal granulocytes, monocytes, and macrophages. These findings suggest that the *c-raf* protooncogene is widely expressed in multiple hematopoietic lineages.

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CERTAIN PROTOONCOGENES have been implicated in the regulation of human hematopoietic cell proliferation and differentiation. Expression of the *c-myc* and *c-myb* protooncogenes has been associated with myeloid cell growth.[1-4] *c-myc* and *c-myb* transcripts also decrease during induction of granulocytic and monocytic differentiation.[1-8] In contrast, the transforming *N-ras* oncogene is expressed at similar levels during both myeloid proliferation and differentiation.[8]

Monocytic differentiation of myeloid cells has been associated with the induction of *c-fos*, *c-fms*, and *c-sis* expression.[5,8,9] The transient appearance of *c-fos* RNA during differentiation along the monocytic lineage precedes the induction of *c-fms* and *c-sis* transcripts. Macrophages thus express *c-sis* RNA and a platelet-derived growth factor-like molecule.[10,11] Furthermore, the *c-fms* gene product is a tyrosine kinase that is related to the receptor for the mononuclear colony-simulating factor CSF-1.[12]

The present study has similarly monitored the expression of *c-raf* in human hematopoietic cells. *V-raf* is the transforming gene of the murine sarcoma virus 3611.[13] This virus induces fibrosarcomas in newborn mice and transforms both fibroblasts and epithelial cells in culture.[13] A recombinant murine retrovirus carrying both the *raf* and *myc* oncogenes induces immunoblastic lymphomas of T and B lineage cells.[14] Infection of murine macrophages with the *raf/myc* recombinant virus also causes growth factor independence and immortalization.[15] Furthermore, *c-raf*-specific RNA has been detected in T cells of an autoimmune strain of mice and in tumors of B cell lineage.[16] These findings have suggested an association between *raf* expression and certain hematopoietic tumors. The presence of *c-raf* transcripts in normal and transformed hematopoietic cells derived from other lineages, however, is required to determine whether expression of this protooncogene is restricted to specific tumors.

The present results demonstrate that (1) *c-raf* is expressed in human HL-60 promyelocytic leukemic cells, (2) the level of *c-raf* transcripts is unrelated to HL-60 growth and differentiation, (3) *c-raf* RNA is not superinducible with cycloheximide, and (4) *c-raf* is detectable in hematopoietic cells differentiated along the granulocytic, monocytic, and lymphoid lineages.

MATERIALS AND METHODS

Cell lines and peripheral blood cells. The HL-60 promyelocytic leukemia cell line, the KG1 myeloid cell line, the U937 monocytic cell line, the NA and AG-876 Burkitt cell lines, the JY B cell line, the MOLT-4 T cell line, and the BeWo and JEG human choriocarcinoma cell lines were maintained in logarithmic growth phase. The HL-60 cells were also grown in the presence of 1.25% dimethyl sulfoxide (DMSO) (Fisher Scientific Co, Boston), 4 mmol/L hexamethylene bisacetamide (HMBA) (Sigma Chemical Co, St Louis), 3.3×10^{-8} mol/L (TPA) (Sigma), and 5×10^{-7} mol/L $1{,}25(OH)_2D_3$. Cytocentrifuge smears of induced HL-60 cells were examined for the expression of alphanaphthyl acetate esterase and nitroblue tetrazolium reduction.[17] Adherence was monitored by cellular attachment to the tissue culture flask. The percentage of positive cells was determined by counting 200 cells in duplicate. Granulocytes, monocytes, and macrophages were isolated as previously described.[18]

DNA probes. The *c-myc*, *c-fms*, *N-ras*, and actin inserts were isolated from their vector sequences as previously described.[8] The 0.7-kb *EcoRI-SalI* fragment of the *v-raf* gene was purified from the p171 plasmid.[13]

RNA extraction and hybridization. The procedures used for RNA extraction, transfer, hybridization, and washing were as reported previously.[8]

RESULTS

We have used the HL-60 promyelocytic cell line to study *c-raf* expression during proliferation and induction of hema-

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topoietic cell differentiation. HL-60 cells were induced along the monocytic lineage after treatment with TPA[19] or 1,25$(OH)_2D_3$.[20] In contrast, these cells were also induced to differentiate along the granulocytic pathway by treatment with DMSO or HMBA.[1,21] HL-60 cells were collected at 24 and 72 hours after treatment with each of these inducers to monitor for *c-raf* expression. A 3.6-kb *c-raf* transcript was detectable in uninduced HL-60 cells (Fig 1). Furthermore, the level of *c-raf* RNA was comparable following induction along both the monocytic and granulocytic lineages. The similar levels of *c-raf* RNA during HL-60 proliferation and differentiation were comparable with the findings obtained with the actin probe (Fig 1).

These findings were extended by examining earlier time points after treatment of HL-60 cells with TPA. TPA (3.3×10^{-8} mol/L) exposure of HL-60 cells resulted in an immediate cessation of growth. The level of *c-myc* transcripts declined by six hours, whereas *c-fms* RNA was detectable by 12 hours (Fig 2). In contrast, there was no detectable difference in the level of *c-raf* transcripts (Fig 2), a finding similar to that obtained when using the *N-ras* probe[7] (Fig 2). Furthermore, although cycloheximide superinduced the expression of *c-myc* and inhibited the induction of *c-fms* RNA, the inhibition of protein synthesis had no detectable effect on *c-raf* expression (Fig 2).

The detection of *c-raf* transcripts in HL-60 cells induced along the monocytic and granulocytic pathways suggested that this gene is expressed in multiple lineages. Indeed, *c-raf* RNA was also detectable in human U937 monocytic leukemia cells, U937 cells induced with TPA, and human peripheral blood monocytes (Fig 3A). In addition, *c-raf* transcripts were also detectable in human granulocytes, macrophages, and KG-1 myeloid leukemia cells (Fig 3B). Furthermore, Burkitt cell lines (NA, AG-876), the JY B cell line, and MOLT-4 T cell line also expressed the 3.6-kb *c-raf* RNA. Finally, *c-raf* transcripts were also detectable in trophoblastic cell lines derived from choriocarcinomas (BeWo, JEG).

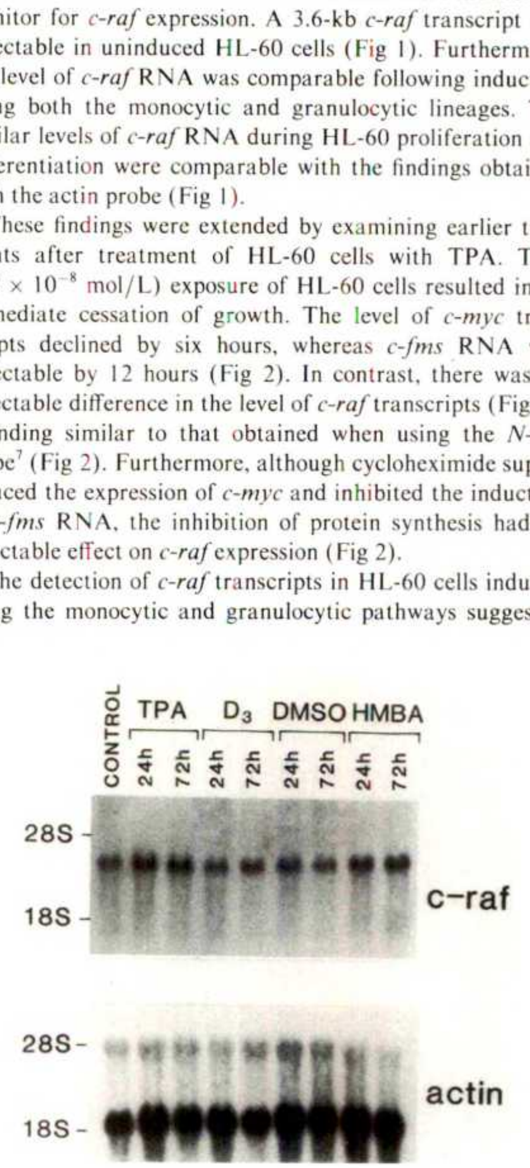

Fig 1. Levels of *c-raf* RNA during induction of HL-60 monocytic (TPA and 1,25$(OH)_2D_3$) and granulocytic (DMSO and HMBA) differentiation. HL-60 cells were grown as previously described[21] in the presence of 3.3×10^{-8} mol/L TPA, 5×10^{-7} mol/L 1,25$(OH)_2D_3$, 1.25% DMSO, or 4 mmol/L HMBA for 24 and 72 hours. Total cellular RNA was purified by the guanidine thiocyanate–cesium chloride method, analyzed by electrophoresis of 15 μg RNA through 1% agarose-formaldehyde gels, and followed by Northern blot transfer to nitrocellulose.[8] The RNA filters were hybridized for 20 hours at 45°C with 10^6 cpm of ^{32}P-labeled nick-translated probe per milliliter of hybridization buffer. The 0.7-kb *EcoRI/SalI* fragment of the *v-raf* gene was purified from the p171 plasmid. The 2.0-kb *PstI* fragment of the chicken β-actin gene was purified from the pA1 plasmid. Cytocentrifuge smears of HL-60 cells were examined after 72 hours of continuous drug exposure to confirm induction of differentiation.[8]

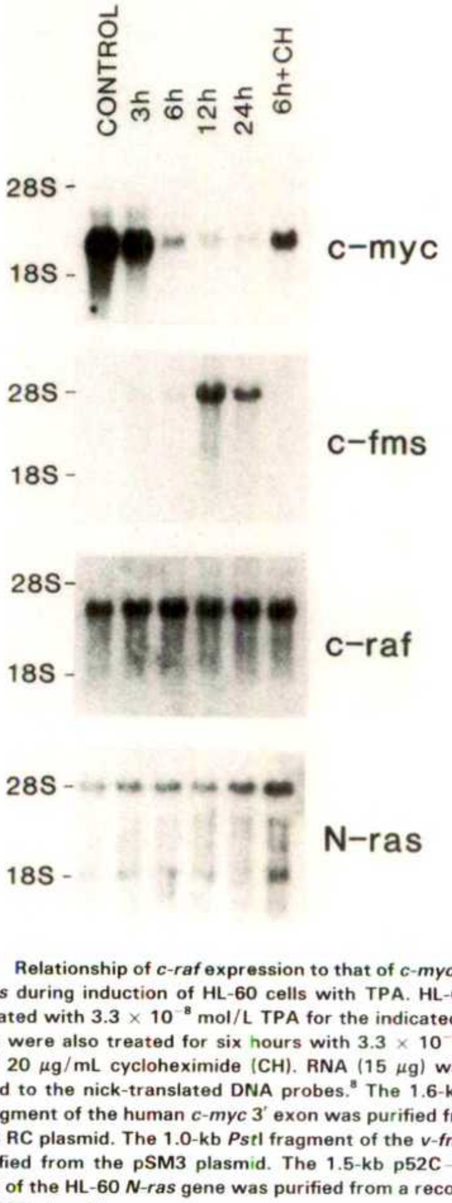

Fig 2. Relationship of *c-raf* expression to that of *c-myc, c-fms,* and *N-ras* during induction of HL-60 cells with TPA. HL-60 cells were treated with 3.3×10^{-8} mol/L TPA for the indicated times. The cells were also treated for six hours with 3.3×10^{-8} mol/L TPA and 20 μg/mL cycloheximide (CH). RNA (15 μg) was then hybridized to the nick-translated DNA probes.[8] The 1.6-kb *ClaI/EcoRI* fragment of the human *c-myc* 3′ exon was purified from the pMC41-3 RC plasmid. The 1.0-kb *PstI* fragment of the *v-fms* gene was purified from the pSM3 plasmid. The 1.5-kb p52C– *EcoRI* fragment of the HL-60 *N-ras* gene was purified from a reconstruction pBR322 plasmid.

DISCUSSION

Two human genes are homologous to *v-raf*.[22] *C-raf*–2 is a processed pseudogene located on chromosome 4, whereas the location of *c-raf*–1 on chromosome 3 has suggested that this gene may be involved in certain human tumors characterized

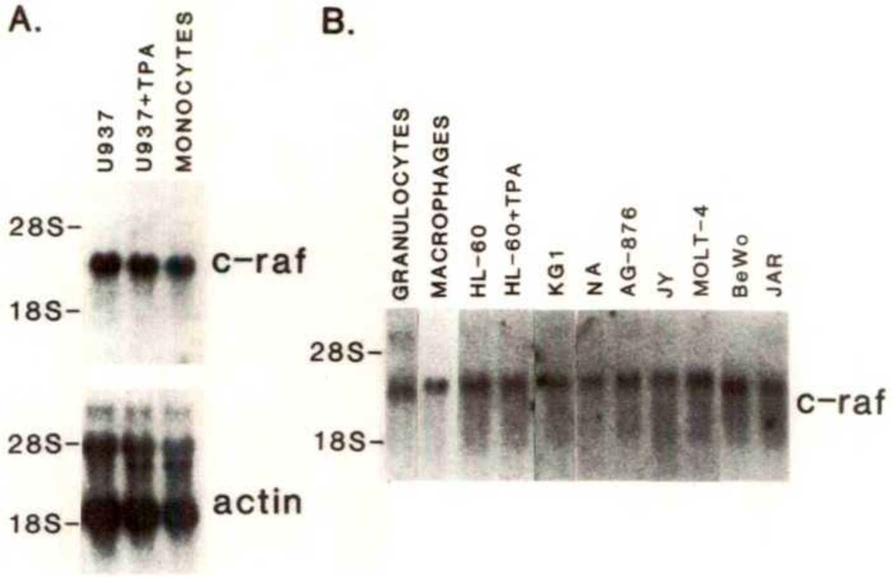

Fig 3. *C-raf* **transcripts in human peripheral blood cells and cell lines. The ^{32}P-labeled** *c-raf* **and actin probes were hybridized to RNA obtained from the various cell preparations (B). The U937 cells were treated with 3.3 × 10^{-8} mol/L TPA for 24 hours (A). Peripheral blood monocytes, granulocytes, and macrophages were purified as described.[18] The methods were as described previously[8] and in the legend to Fig 1. For purposes of comparison, the intensities of the** *c-raf* **hybrids shown in (A) were equivalent to that obtained for HL-60 cells run in the same experiment.**

by chromosomal aberrations in this region.[22] Indeed, *c-raf*-specific RNA has been detected in human small cell lung carcinomas but not in normal bronchial epithelial cells.[23] An activated *c-raf* gene has also been demonstrated in a human gastric carcinoma[24] and a glioblastoma.[25] The mechanism of *c-raf* activation is probably related to a promotor insertion mechanism or expression of a truncated gene product.[26,27]

The present results demonstrate that *c-raf* transcripts are detectable in hematopoietic cells from multiple lineages. The level of *c-raf* transcripts is independent of both hematopoietic cell growth and differentiation. Furthermore, in contrast to *c-myc* and *c-fms*,[8] regulation of *c-raf* expression is unaffected by inhibition of protein synthesis. The results also suggest that cell lines derived from myeloid and lymphoid tumors are not associated with inappropriately high levels of *c-raf* expression or a different sized transcript when compared with normal hematopoietic cells. Taken together, these findings might suggest that the *c-raf* gene product is involved in events other than either growth control or differentiation of normal and transformed hematopoietic cells.

The role of the *c-raf* gene product is unknown. The predicted amino acid sequence of *v-raf* has revealed significant homology with the tyrosine kinase–encoding oncogene family that in part includes *v-src*, *v-abl*, *v-fes*, and *v-fms*.[22] In contrast to *c-src* and *c-abl*, which are expressed in both hematopoietic and nonhematopoietic cells,[28,29] *c-fes* and *c-fms* protooncogene expression has been specifically implicated in myelopoiesis.[8,12,30] Of interest, induction of HL-60 cells along the granulocytic or monocytic lineages is associated with the activation of pp60$^{c\text{-}src}$ kinase activity but not with an increase in *c-src* RNA levels.[31,32] Thus, although *c-raf* transcripts are detectable in hematopoietic cells and cell lines of different lineages, a quantitative difference at the product level or a qualitative difference in the *c-raf* protein may play a role in hematopoietic differentiation along specific pathways. Alternatively, the *c-raf* gene product may act by phosphorylating proteins involved in mechanisms unrelated to specific hematopoietic cell function. In this regard, further studies will be required to determine whether *c-raf* expression in choriocarcinoma cell lines is related to this transformed phenotype and whether the *c-raf* gene is specifically expressed in certain other normal tissues of nonhematopoietic origin.

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Comparison of Three Remission Induction Regimens and Two Postinduction Strategies for the Treatment of Acute Nonlymphocytic Leukemia: A Cancer and Leukemia Group B Study

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Samuel Kopel, Robert N. Levy, Robert Carey, Phillip Schulman, Arlan J. Gottlieb, O. Ross McIntyre,
and the Cancer and Leukemia Group B

Patients with acute nonlymphocytic leukemia were randomized to receive remission induction therapy consisting of seven days of cytosine arabinoside and three days of daunorubicin ("7 + 3") or to receive the same regimen intensified by either the addition of 6-thioguanine or by extension of the administration of cytosine arabinoside to ten days. Additionally, all patients were randomized to receive or not to receive cotrimoxazole antibacterial prophylaxis during the remission induction phase. Neither an increase in intensity of chemotherapy nor the antibacterial prophylaxis increased the remission rate above the 53% for patients treated with the standard "7 + 3" regimen.

The second part of this study addressed the issue of the utility of long-term maintenance chemotherapy. To this end, patients were randomized to discontinue all treatment after 8 months of maintenance chemotherapy or to continue maintenance therapy for a total of 3 years. Although there was a transient increase in the relapse rate for patients who discontinued therapy, the proportion of long-term remitters was identical in the two patient groups. Additionally, there is a suggestion of a survival advantage for patients randomized to discontinue all therapy at 8 months.

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THE TREATMENT of acute nonlymphocytic leukemia (ANLL) consists of a remission induction phase designed to return hematopoiesis to normal and a postinduction phase designed to prolong the remission. The first part of the study to be reported here was designed to determine whether an increase in the intensity of the standard seven days of cytosine arabinoside and three days of daunorubicin (DNR) ("7 + 3") remission induction regimen used by the Cancer and Leukemia Group B (CALGB) since 1974[1,2] and/or the administration of cotrimoxazole antibacterial prophylaxis[3-5] would result in an increase in the complete remission (CR) rate. The second phase of the study was designed to determine whether prolonged maintenance therapy of moderate intensity during the postinduction phase was necessary.[6] Accordingly, all patients received maintenance chemotherapy for 8 months, at which time half were randomized to discontinue all therapy and the other half to continue monthly courses of maintenance therapy for a total of 3 years.

The data described in this paper demonstrate that neither an increase in the intensity of 7 + 3 therapy, as described here, nor the administration of cotrimoxazole results in an improvement in the CR rate and that maintenance chemotherapy beyond 8 months is unnecessary. In addition, data are presented that reinforce an earlier hypothesis that leukemic relapse may occur as a result of at least two distinct biologic processes.[7,8]

MATERIALS AND METHODS

Study Design

CALGB study 7921 was activated in November 1979 and was closed to accrual in November 1982.

Eligibility

Any patient with acute nonlymphocytic leukemia (ANLL) according to the criteria established by the French-American-British (FAB) working party was eligible for study.[9] Patients with life-threatening infections were eligible only upon control of such infections. Informed consent was required prior to entry into the study.

Patients were ineligible if they had inadequate renal function. Although the protocol specified maximum permissible levels of 30 mg/dL for BUN and 1.5 mg/dL for creatinine levels, a number of patients were entered who had levels slightly above these limits. Although technically ineligible, these patients were included in the study, with careful analyses being performed to determine whether they showed any differences in response or toxicity. Any patient whose BUN was >40 mg/dL or whose creatinine level was >2.0 mg/dL was excluded from analysis. Patients with a history of neoplastic disease and associated cytotoxic therapy were not eligible for randomization (See Statistical Methods).[10,11]

Treatments Under Study

Remission induction therapy. Figure 1A provides the schema for the induction phase of the study. Patients were initially randomized to induction therapy with the standard 7 + 3 regimen (cytosine arabinoside, 100 mg/m^2/d by continuous infusion for seven days, and DNR, 45 mg/m^2 on days 1, 2, and 3) or with induction therapy

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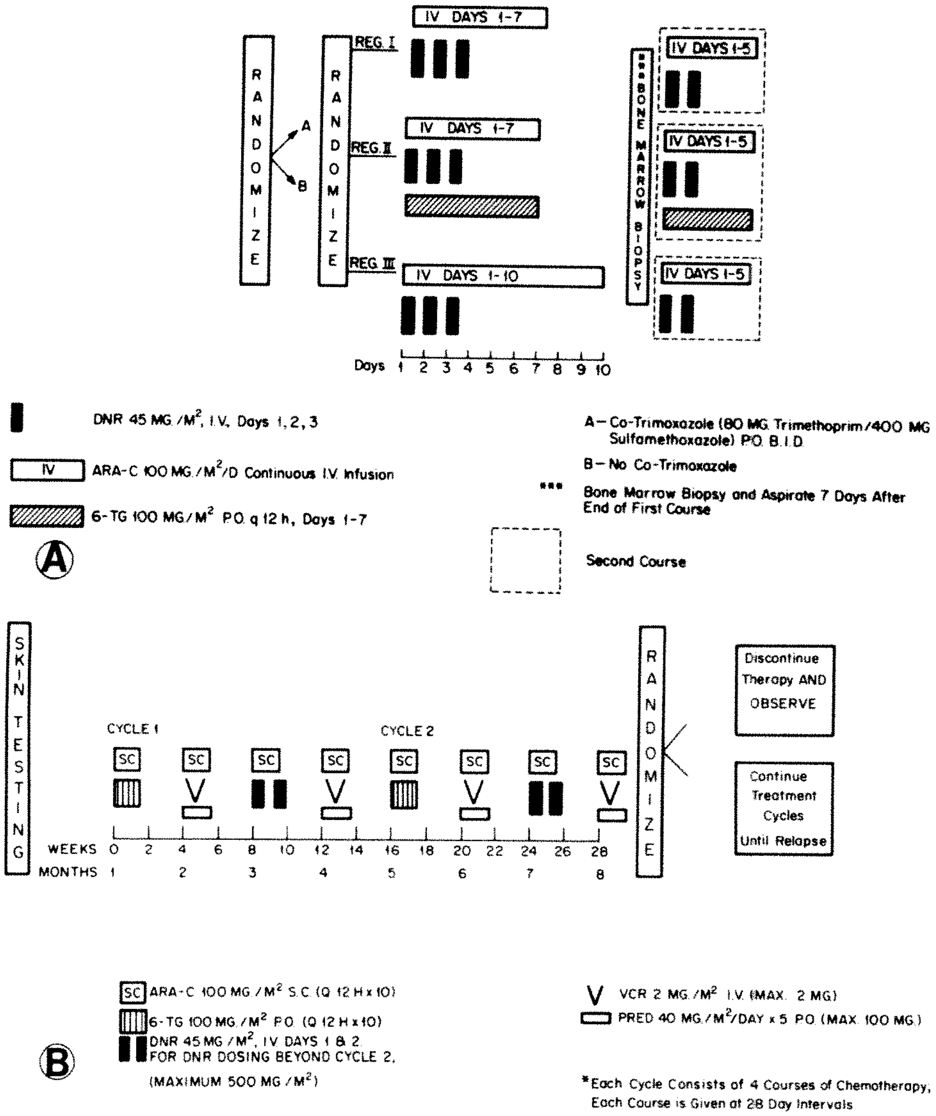
RANDOMIZE
A
B
RANDOMIZE
REG. I
IV DAYS 1-7
REG. II
IV DAYS 1-7
REG. III
IV DAYS 1-10
Days 1 2 3 4 5 6 7 8 9 10
*** BONE MARROW BIOPSY
IV DAYS 1-5
IV DAYS 1-5
IV DAYS 1-5
DNR 45 MG/M², I.V. Days 1, 2, 3
IV ARA-C 100 MG/M²/D Continuous I.V. Infusion
6-TG 100 MG/M² P.O. q 12 h, Days 1-7
A— Co-Trimoxazole (80 MG Trimethoprim/400 MG Sulfamethoxazole) P.O. B.I.D.
B— No Co-Trimoxazole
*** Bone Marrow Biopsy and Aspirate 7 Days After End of First Course
Second Course
A
SKIN TESTING
CYCLE 1
CYCLE 2
SC
V
WEEKS 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
MONTHS 1 2 3 4 5 6 7 8
RANDOMIZE
Discontinue Therapy AND OBSERVE
Continue Treatment Cycles Until Relapse
SC ARA-C 100 MG/M² S.C. (Q 12 H x 10)
6-TG 100 MG/M² P.O. (Q 12 H x 10)
DNR 45 MG/M², IV DAYS 1 & 2 FOR DNR DOSING BEYOND CYCLE 2, (MAXIMUM 500 MG/M²)
V VCR 2 MG/M² I.V. (MAX. 2 MG)
PRED 40 MG/M²/DAY x 5 PO (MAX. 100 MG.)
*Each Cycle Consists of 4 Courses of Chemotherapy, Each Course is Given at 28 Day Intervals
B

Fig 1. Treatment schema. (A) Remission induction phase. (B) Maintenance phase.

whose intensity was increased either by extending the administration of cytosine arabinoside to ten days ("10 + 3") or by the addition of 6-thioguanine (6-TG; TAD) at 100 mg/m^2 every 12 hours for seven days concurrent with cytosine arabinoside. These two modifications of the 7 + 3 regimen were based upon reports in the literature that the therapeutic efficacy of our standard regimen might be improved by extending cytosine arabinoside administration to ten days[12] or by the addition of 6-TG.[13-15]

A second course of therapy was administered, as indicated in Fig 1A, if the patient's leukemia failed to enter remission. The only exceptions were patients who achieved a partial remission and in whom the severity of toxicity during the first course of therapy was felt to preclude the administration of course 2. Half of the patients on each program were randomized to receive cotrimoxazole, two regular-strength tablets (80 mg trimethoprim and 400 mg sulfamethoxazole), by mouth twice a day starting on the day before initiation of chemotherapy and continuing until remission occurred or until the patient was removed from the study as a treatment failure.

The study was amended in November 1980 so that the DNR dose in all three regimens was reduced to 30 mg/m^2/d for patients over 60 years old. This modification was introduced as a consequence of the previous CALGB protocol in ANLL that suggested that this change would increase the CR rate and reduce the death rate for older patients.[2]

Patients who entered CR or partial remission following the second course of induction therapy advanced to the maintenance phase. The remaining patients were considered to be treatment failures and were removed from the study.

Dose reductions of 50% and/or dose interruptions were permitted for severe gastrointestinal toxicity, hepatic dysfunction, or renal dysfunction. A lumbar puncture was performed on the day prior to the initiation of chemotherapy and every 6 months for 5 years. If any leukemic cells were seen on a cytocentrifuge preparation, the patient received 50 mg of cytosine arabinoside intrathecally every four days until leukemic cells were no longer detectable in the CSF, and this was followed by the same dose of cytosine arabinoside every 4 weeks for 8 months.

Maintenance therapy. As indicated in Fig 1B, maintenance therapy consisted of a series of monthly five-day courses of combination chemotherapy. Each cycle was composed of four such courses. Sequential courses consisted of five days of administration of cytosine arabinoside together with 6-TG (course 1), prednisone/vincristine (courses 2 and 4), or DNR (course 3) administered on a rotating schedule. When a cumulative dose of 500 mg/m^2 of DNR was reached, the third course of each cycle was modified by replacement of DNR with 6-TG, 100 mg/m^2 orally every 12 hours for ten times.

Following two cycles of maintenance therapy (8 months), marrow aspiration and biopsy were performed. Patients who were still in CR at this time were randomized either to stop maintenance chemotherapy immediately or to continue maintenance for an additional seven cycles (for a total duration of maintenance therapy of 3 years) or until the time of relapse if that occurred before the seven cycles were completed.

Bone marrow aspiration was repeated every 4 months until relapse for all patients regardless of their maintenance treatment assignment.

Evaluation of Response

A CR was defined by the criteria previously reported by Rai et al.[1] In brief, a bone marrow aspirate had to contain $<5\%$ abnormal cells, with a normal cellularity and normal granulopoiesis, thrombopoiesis, and erythropoiesis. Additional requirements included a peripheral blood count within normal limits and clearing of all signs and symptoms attributable to leukemia.

Relapse was defined as a return of leukemic cells to the marrow in excess of 25%.

Statistical Methods

Patients who were clearly ineligible were excluded from this analysis. Patients who were registered but withdrawn prior to the initiation of protocol therapy and patients with no follow-up data after registration were also excluded. Patients with prior cancer were not eligible for randomization but were nonrandomly assigned to treatment with 7 + 3 without cotrimoxizole and have been excluded from this report. Seven hundred forty-nine patients were registered in the study. Their study status is shown in Table 1. Six hundred sixty-eight (95.8%) of the 697 patients with primary leukemia were eligible and evaluable.

The primary end points of interest for the induction questions were response rates and duration of survival. Analysis of these end points were stratified by age (60 and under *v* over 60). Comparisons of the three induction regimens were also stratified by the randomized assignment of cotrimoxazole, and the evaluations of this antimicrobial agent were stratified by the induction regimen assigned.

For the maintenance phase of the study, the primary end points were time to relapse and survival. Both end points were measured from the date of CR and from the time of maintenance randomization (ie, after two cycles of maintenance). These comparisons were stratified by the induction chemotherapy regimen assigned since the maintenance randomization was stratified in that way.

All *P* values reported are two sided. Survival and response duration curves as well as any rates computed from these curves were calculated by using the life table method (product limit estimator) of Kaplan and Meier.[16] Survival comparisons were made using the Mantel-Haenszel (log rank) test.[17] The chi-square test for contingency tables was used for overall comparisons of response rates across strata.[18]

A number of patients were deemed "not evaluable for response," usually as a result of inadequate documentation of marrow status posttreatment. For purposes of estimating and comparing response rates, a conservative approach was used with respect to these patients. They were assumed to have been treatment failures, ie, they were included in the denominators of the rates, but not in the numerators.

A number of patients entered bone marrow transplantation programs prior to completion of this study. Patients who entered transplantation programs while in their initial remission were considered censored at the date of entry into that program for calculation of the remission duration but continued to be followed for survival.

Toxicity of the treatments under study was assessed by using previously reported CALGB toxicity criteria.[2] Specific comparisons of infection rates between the cotrimoxazole-treated and the control groups were used to determine the efficacy of prophylactic administration of cotrimoxazole.

RESULTS

Remission Induction Therapy

The Outcome of Remission Induction Therapy

Fifty-six percent of the 668 eligible patients entered CR, 4% achieved a partial remission, 24% expired during therapy, 14% survived but did not enter remission, and 1% were inevaluable because of a lack of definitive marrow studies. The CR rates for 7 + 3, TAD, and 10 + 3 regimens were 52.6%, 56.9%, and 57.3%, respectively (Table 2) ($P = .6$). Fewer patients treated with 10 + 3 required two courses of therapy to enter remission (15% of complete responders) than did patients treated with 7 + 3 (29%) or TAD (28%) ($P = .011$), but the median time to remission was not significantly different for the three regimens (10 + 3, 4.8 weeks; 7 + 3, 4.4 weeks; TAD, 4.1 weeks). The toxicity of the three regimens was identical, with all patients experiencing life-threatening hematopoietic toxicity, and 7% to 11% of patients experiencing severe or life-threatening renal, hepatic, and/or cardiac toxicity.

Three hundred twenty-three patients were randomized to receive cotrimoxazole, and 345 patients were randomized to receive no antimicrobial prophylaxis during remission induction therapy. The CR rate was 56% whether or not the patient received cotrimoxazole. In addition, cotrimoxazole

Table 1. Study Status of Patients in the Study

	With Cotrimoxazole			Without Cotrimoxazole			
	7 + 3	TAD	10 + 3	7 + 3	TAD	10 + 3	Total
Patients entered							749
Prior cancer							52
Patients randomized	106	110	119	116	111	135	697
Ineligible	1	2	2	5	2	5	17
Cancelled	1	1	3	1	0	1	7
No follow-up	2	0	0	1	0	2	5
Cases analyzed	102	107	114	109	109	127	668

Table 2. Outcomes of Remission Induction Therapy

	"7 + 3"	TAD	"10 + 3"
Number of patients	211	216	241
CR	111 (53)*	123 (57)	138 (57)
Partial remission†	13 (6)	6 (3)	10 (4)
No response alive†	38 (18)	31 (14)	26 (11)
Expired‡	45 (21)	55 (26)	63 (26)
Not evaluable	4 (2)	1 (0.5)	4 (2)

*Number of patients (%).

†Patients whose leukemia proved to be resistant to the chemotherapy administered.

‡Death during remission induction therapy.

had no effect on patient survival or on the toxicity associated with each of the chemotherapy treatment arms.

Prognostic Factors for the Outcome of Remission Induction Therapy

Table 3 lists the patient characteristics that were evaluated for possible relationship to the outcome of remission induction therapy. Only five of these 20 characteristics were significantly related to treatment outcome, and one was of borderline significance (Table 4). Age was by far the single most important factor, with only 41% of 104 patients >60 years old entering remission as compared with 65% of patients ≤ 60 years old ($P < .00001$). The next most important factor was serum creatinine levels. Only 36% of patients with a creatinine level of 1.6 to 2.0 mg/dL entered remission as compared with 56% for patients whose creatinine level was <1.6 ($P = .006$). An SGOT level of ≥ 40 mU/mL was associated with a CR rate of 47% compared with 59% for patients whose SGOT level was <40 mU/mL. A WBC count at diagnosis of $>100{,}000/\mu$L was associated with a CR rate of only 38% as compared with 58% for patients with a count below this value ($P = .041$). The presence of skin or gingival involvement was an unexpectedly good prognostic sign, being associated with a CR rate of 68% as compared with a CR rate of 54% for patients without such extramedullary involvement ($P = .032$).

Forty-three percent of patients presented without fever or infection. These patients tended to have a higher CR rate than the 57% of patients who were febrile and/or had a documented infection at the time of diagnosis (60% *v* 52.6% CR rates respectively, $P = .07$). The presence of severe infection was also of prognostic significance, being associated with a CR rate of only 40% as compared with a CR rate of 57% for patients without severe infection ($P = .057$). The administration of cotrimoxazole had no effect on the outcome of treatment or on the survival of patients whether or not they were afebrile or febrile, infected or not infected at diagnosis. The incidence of severe or life-threatening infection during induction therapy for patients who were afebrile at diagnosis was reduced from 73% to 59% by the administration of cotrimoxazole ($P = .013$), but as noted earlier, this had no effect on treatment outcome. Table 5 provides information regarding the types of infection experienced by patients who received or did not receive cotrimoxazole. Bacterial infections were the predominant cause of first infections. Fungal infections were frequent causes of second infections and were more common in the cotrimoxazole group (50%) than in the control group (29%).

Table 3. Patient Characteristics at Diagnosis Evaluated for Potential Prognostic Significance

Age
Sex
Race

WBC count
Number of circulating leukemic cells
Hematocrit
Hemoglobin level
Platelet count
Bleeding
Temperature

Infection
CNS involvement
Skin or gingival involvement
Hepatomegaly
Splenomegaly
Lymphadenopathy
BUN
Creatinine
SGOT
Bilirubin

Table 4. Factors of Prognostic Significance

	Remission Induction Outcome	Remission Duration	Survival
Age	<.00001	.023	<.0001
WBC count	.041	NS	.013
Infection	.057	NS	.008
Skin or gingival involvement	.032	NS	NS
Creatinine	.006	NS	.016
SGOT	.018	NS	.0001
Hemoglobin	NS	.001	.008

Results are *P* values using univariate analysis.

Abbreviation: NS, not significant.

Miscellaneous Observations Regarding the Outcome of Remission Induction Therapy

One year after the study was initiated, the protocol was modified so that patients >60 years of age would receive 30 mg/m^2/d of DNR rather than 45 mg/m^2/d. One hundred eleven patients in this age group received the higher DNR dosage, whereas 139 patients received the lower dosage. Although the CR rates were not significantly different (39% *v* 43% respectively), fewer patients treated at the lower dosage died during therapy (31% *v* 45% respectively, $P = .028$).

Table 5. Types of Induction Infections

	With Cotrimoxazole (n = 323)		Without Cotrimoxazole (n = 345)	
	Severe* Infection	Life-Threatening Infection†	Severe Infection	Life-Threatening Infection
First induction infection‡				
Bacterial	14	20	14	40
Fungal	6	11	5	9
Viral	3	0	1	1
Multiple	2	3	1	5
FUO	37	5	49	6
Type not reported	54	25	66	13
Second induction infection				
Bacterial	3	20	4	28
Fungal	9	14	6	7
Viral	0	0	1	0
Multiple	0	3	0	3
FUO	4	0	7	1
Type not reported	19	5	23	12

Abbreviation: FUO, fever of undetermined origin.

*Local or visceral infection with systemic signs and/or a fever of 38°C.

†Fulminant local or visceral infection with sepsis, shock, or other system failure.

‡Type of infection documented by culture or by biopsy.

Remission Duration

Figure 2A provides the remission duration curve for all patients who entered CR. The median was 52.4 weeks; 22% of patients were projected to be in remission at 3 years. There was no statistically significant difference in remission duration between patients treated on the three induction arms. The overall remission duration was related to the number of courses necessary to induce a CR. Patients who required a single course of therapy had longer remissions than those who required two courses (median remission durations of 55.9 *v* 45.4 weeks respectively, $P = .02$). Patients who entered partial remission status relapsed at a median of 20.7 weeks, with no patient remaining in this status beyond 100 weeks.

One hundred forty-eight patients relapsed or died before reaching the point of second randomization to stop or to continue maintenance therapy. Of the remaining patients, 151 were randomized, and 73 were not. There were no statistically significant differences in remission duration between patients who were or were not randomized. The median duration of remission for all patients after randomization was 67 weeks, with 38% of patients projected still to be in remission 3 years later (Fig 2b). This figure also provides the remission duration curve for patients randomized to continue or to stop therapy. The curves diverged 3 months after randomization but converged and became persistently superimposable 75 weeks after randomization. The median time to relapse after randomization for patients in whom therapy was discontinued was 45 weeks compared with 71 weeks in patients who continued therapy ($P = .62$). The type of remission induction therapy had no statistically significant effect on the time to relapse for patients randomized to stop or to continue maintenance chemotherapy.

Toxicity Associated With Maintenance Chemotherapy

Toxicity data were available for 329 patients for the first cycle of maintenance therapy and for 220 patients on the second cycle. Seventy-five percent of patients experienced severe or worse hematologic toxicity at some time during cycle 1 with a reduction of the blood counts to life-threatening levels (WBC count reduced to $<1{,}000/\mu L$, granulocyte count to $<500/\mu L$, and/or platelet count to $<25{,}000/\mu L$) occurring in 51% of patients. Sixty-six percent of patients who received cycle 2 of therapy had severe hematologic toxicity, with 31% having life-threatening reductions in blood counts to the levels just described.

Death in remission occurred only during cycles 1 and 2 and was clearly age related, with a 3% death rate in remission for patients <40 years old, 9% for patients 41 to 60 years old, and 14% for patients >60 years old ($P = .03$).

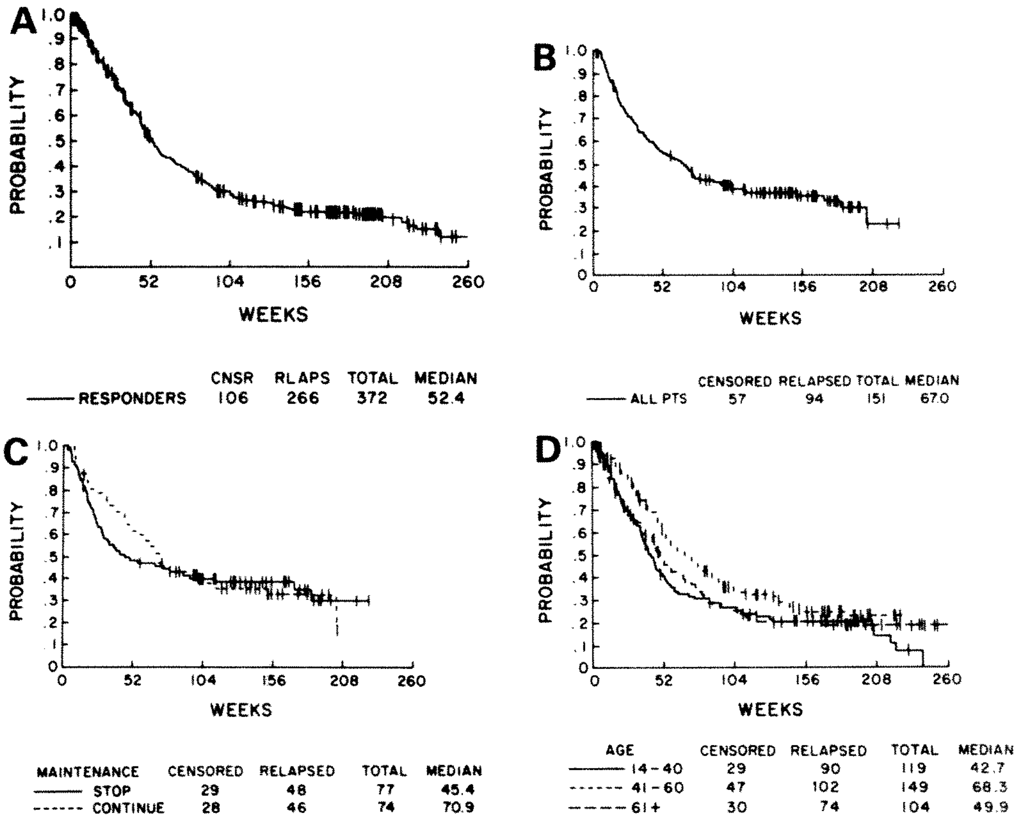

Fig 2. Remission duration. (A) Remission duration curve for all patients whose leukemia entered CR. (B) Remission duration subsequent to randomization to stop or continue maintenance therapy—all patients—and (C) relationship of remission duration to the cessation or continuation of chemotherapy beyond 8 months. (D) Relationship between age and remission duration.

Clinical Prognostic Factors for Remission Duration

Of the various factors evaluated, only age at diagnosis and initial hemoglobin level were statistically significantly prognostic factors for remission duration (Table 4). Figure 2C demonstrates that patients between 41 and 60 years of age had the longest remission durations (median, 68.3 weeks). Older and younger individuals had significantly shorter remissions (medians of 49.9 and 42.7 months respectively, $P = .026$). Patients with a hemoglobin value of 8 to 12 g/dL had a median remission duration of 57.6 weeks; those with hemoglobin values <8 had a median of 51 weeks, and those with hemoglobin values of 12 to 18 had a median of 36 weeks ($P = .002$).

Survival

Figure 3A presents the survival curve for the 668 evaluable patients. The median duration of survival is 41.2 weeks; 18% of patients are alive at 3 years. The survival curve has three components, each with a characteristic slope. The first component, which has the steepest slope, represents death during remission induction therapy. The second component represents death subsequent to relapse during the first 2 years of remission, and the third component represents late relapses and death. As noted earlier, the only deaths seen in remission occurred during the first two cycles of maintenance therapy and were age related.

Table 4 lists the factors that were prognostically important for survival. From the data provided it can be seen that the majority of factors were important because of their relationship to the outcome of remission induction therapy. Figure 3B illustrates this effect. Note that as the severity of infection increases, the fall in the first part of the survival curve becomes greater, thus indicating increasing mortality during remission induction therapy. Once the period of induction

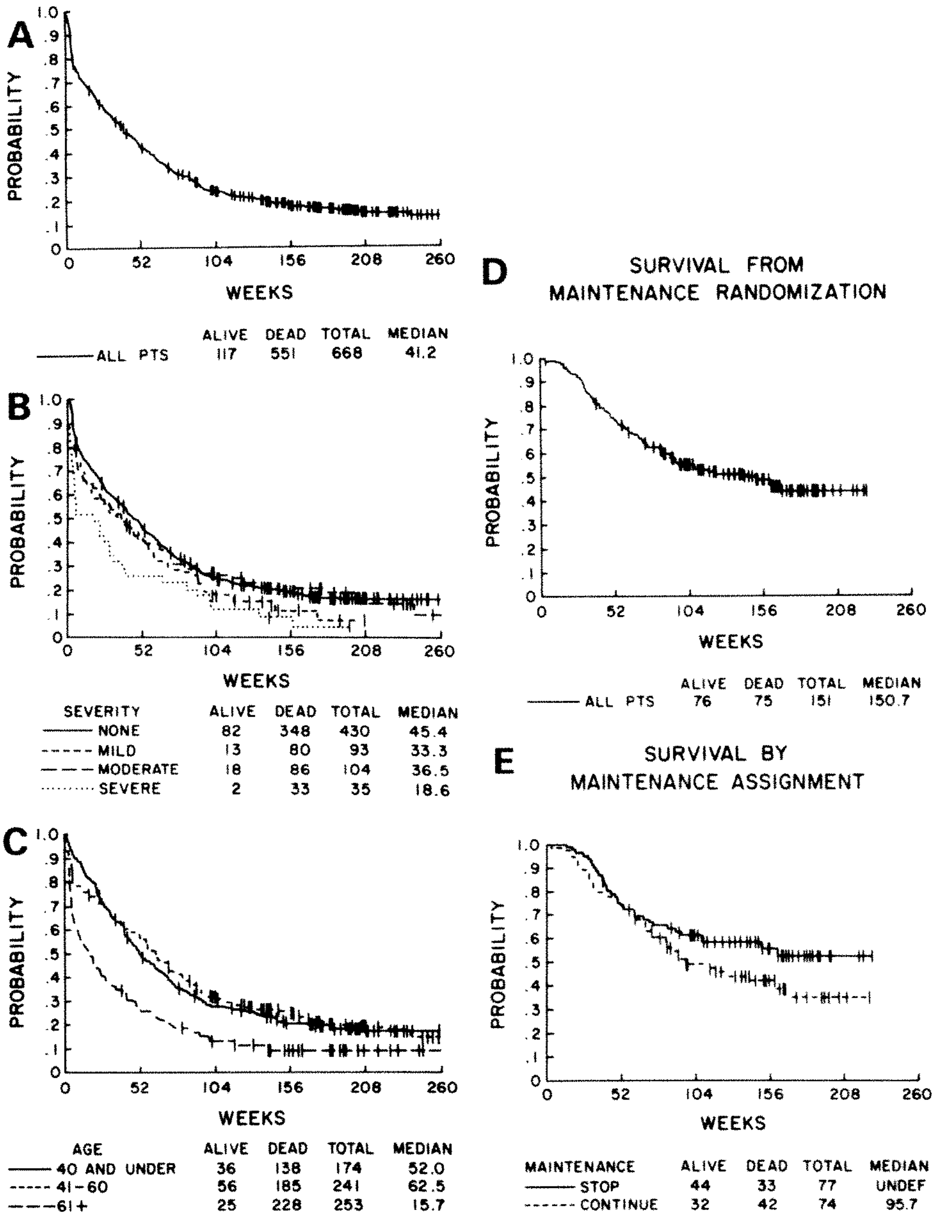

Fig 3. Survival. (A) Survival curve for all patients entered into this study. (B) Relationship between severity of infection and survival: mild infection, mild cutaneous or upper respiratory infection without fever; moderate infection, evidence of infection with temperature >38°C; severe infection, local or visceral infection with systemic signs and/or temperature >38°C. (C) Relationship between age and survival. (D) Survival after randomization—all patients—and (E) survival after randomization by treatment arm.

death is passed, all the curves are parallel, which indicates that infection at diagnosis has no effect on events subsequent to the patient entering remission. The hemoglobin level, on the other hand, is prognostically important because of its relationship to remission duration. In this case, the survival curves for patients with different hemoglobin levels at diagnosis are virtually identical for the first phase of the curve but diverge thereafter (data not shown). The survival curves for patients treated with the three different remission induction regimens were virtually identical.

In contrast, age was of prognostic importance with respect to both the outcome of remission induction therapy and remission duration. Figure 3C demonstrates that patients <40 years old are more likely to survive remission induction therapy than are older patients, whereas patients over 60 years of age are not only more likely to die early in therapy but also have a higher death rate during the first year of remission. When these data are considered together with the data presented in Fig 2C, it is apparent that older patients are at a disadvantage because of a higher relapse rate and a higher death rate both during remission induction therapy and subsequent to relapse. Although younger patients also have a higher relapse rate than patients 40 to 60 years old, it is likely that their greater ability to survive after relapse offsets any survival advantage that the patients 40 to 60 years of age might have had from their longer remission durations.

Figure 3D gives the survival curve of the 151 patients randomized to stop or to continue maintenance therapy after the first 32 weeks of maintenance. The median survival time is 150.7 weeks, with 48% of patients projected to be alive at 3 years after randomization. Figure 3D also provides the survival curves for patients randomized to stop or to continue therapy. There appears to be a trend for greater survival for those who stopped therapy, but the current *P* value is .11.

DISCUSSION

The examination of remission induction therapy in this study demonstrated that all three regimens produced the same CR rate. The administration of cotrimoxazole, although reducing the incidence of severe infection, did not improve either the remission induction or survival rates. Evaluation of the types of treatment failure[19,20] demonstrates that almost two thirds of the treatment failures were associated with death during therapy and one third were characterized by persistent leukemia in patients who survived therapy. Given that the former patients might have benefited from a less intensive and therefore less toxic induction regimen and the latter patients might have benefited from more intensive therapy, it is clear that no single alteration in the induction regimens studied here is likely to produce a dramatic increase in the CR rate. Rather, selectivity in treatment with the design of regimens tailored to the needs of the individual patients may be the most likely approach to improve the overall CR rate.

The study reported here failed to demonstrate any therapeutic benefit from the addition of 6-TG to the "7 + 3" cytosine arabinoside/DNR combination. Although others have reported very high remission rates with TAD regimens,[13-15] the doses and schedules used for the cytosine arabinoside and DNR differ from that used. Any differences that might exist in therapeutic efficacy between these TAD regimens and "7 + 3" therapy would appear to be most likely due to differences in the cytosine arabinoside and DNR dose/schedules rather than to the addition of 6-TG. The observations reported here with respect to cotrimoxazole confirm previous observations that the administration of the antimicrobial agent to severely granulocytopenic patients reduces the incidence of severe infections[3-5] and appears to be associated with an increase in fungal infections.[21,22] Perhaps the latter reason together with the fact that cotrimoxazole treatment reduced bacterial infections only to a modest extent accounts for the failure of cotrimoxazole administration to improve the CR rate.

The median duration of remission for the patients treated here was 52.4 weeks, with 22% of patients projected to be in remission at 3 years. In contrast, data presented elsewhere suggest that patients who receive similar remission induction therapy and who do not receive any therapy after having entered remission have median duration of remissions in the range of 7 months.[23-25] The remission duration curves for those patients randomized to stop or to continue maintenance therapy began to diverge approximately 3 months after randomization due to an increase in the relapse rate of those patients in whom therapy was discontinued. The relapse curves then began to converge 6 months after randomization as a result of a significant fall in the relapse rate of those patients whose therapy had been discontinued. The curves became superimposable at 1½ years after randomization and remained so thereafter. Both relapse curves have a point of inflection indicative of a change from a high rate of relapse to a lower relapse rate. This change in relapse rate occurred earlier in patients in whom maintenance therapy was discontinued. Once the lower relapse rate phase began, the continued administration of chemotherapy had no effect on the rate of relapse. Hence, the data reported here demonstrate that the administration of conventional maintenance chemotherapy for 3 years retards leukemic relapse during the first 2 years of administration but has no effect on the rate of relapse thereafter or on the proportion of patients in long-term remission over and above that produced by 8 months of therapy. Patients in whom therapy was discontinued at 8 months appear to have survived longer than patients who receive 3 years of therapy, perhaps because of a survival advantage after relapse.

The relatively modest therapeutic efficacy of maintenance chemotherapy has resulted in the introduction of intensive consolidation chemotherapy.[6] In one Roswell Park Memorial Institute study, three courses of intensive consolidation chemotherapy were administered followed by 3 years of maintenance therapy. The median duration of remission was 17 months, with over 30% of patients remaining in remission beyond 5 years. A successor study, which provided for three or four courses of intensive consolidation therapy and no maintenance therapy, has produced similar results.[23-26] Similar results for intensive consolidation therapy have been reported by other investigators, but the follow-up times have been shorter.[27-29] At the present time CALGB is engaged in a prospective randomized comparison of the efficacy of intensive consolidation chemotherapy.[30] One problem with the intensive consolidation therapies has been that they are associated with significant morbidity, with mortality rates ranging from 5% to 20%.[27-29] Hence, the net beneficial effects of intensive therapy in remission is less than one might expect on the basis of reduced relapse rates. Given recent observations regarding the prognostic significance of the cytogenetic characteristics of the leukemic cells,[31] cell surface markers,[32] and the potential significance of both molecular biologic[33] and cell cycle characteristics of leukemic cells,[34] perhaps pretherapy assessments of these leukemic cell characteristics could be used to identify patients who will have a long remission when treated with maintenance therapy. This would allow these patients to avoid the risks of intensive consolidation therapy.

Age was the most important prognostic factor, with advanced age being associated with a low remission rate. Age >60 or <40 was associated with shorter remissions. The latter observation is curious since a recent review of 760 patients treated with very similar maintenance therapies failed to demonstrate an effect of age on remission duration.[8] An elevated serum creatinine or SGOT level, the presence of infection, and a WBC count greater than 100,000/μL at the time of initiation of treatment were all associated with death during remission induction therapy. Of interest is the unexplained observation that a hemoglobin level between 8 and 12 g/dL was a good prognostic sign with respect to remission duration. Since there was no provision for a central review of slides, no comment can be made regarding the possible relationship between FAB type and response.

The observations reported here also have implications regarding the interpretation of other studies. Therapies such as allogeneic bone marrow transplantation in remission[35-37] may also have their primary effects on the first part of the relapse curve and may be associated with a similar low but significant late relapse rate. Should this prove to be the case, then the hypothesis that at least some late relapses are in fact "new leukemias" may gain support. Finally, the somewhat unexpectedly long subsequent remission durations and survivals of patients who were selected by having remained in remission at 8 months (Figs 2B, 3D) demonstrate the necessity of a control arm in interpreting the efficacy of therapeutic interventions performed several months to years after remission has been attained, be these interventions bone marrow transplantation[35-37] or "late intensification" chemotherapy.[38]

APPENDIX

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Neutrophil-Mediated Protection of Cultured Human Vascular Endothelial Cells From Damage by Growing *Candida albicans* Hyphae

By John E. Edwards, Jr, Daniel Rotrosen, James W. Fontaine, Christian C. Haudenschild, and Richard D. Diamond

Interactions were studied between human neutrophils and cultured human umbilical vein endothelial cells invaded by *Candida albicans*. In the absence of neutrophils, progressive Candida germination and hyphal growth extensively damaged endothelial cell monolayers over a period of 4 to 6 hours, as determined both by morphological changes and release of ^{51}Cr from radiolabeled endothelial cells. Monolayers were completely destroyed and replaced by hyphae after 18 hours of incubation. In contrast, when added 2 hours after the monolayers had been infected with Candida, neutrophils selectively migrated toward and attached to hyphae at points of hyphal penetration into individual endothelial cells (observed by time-lapse videomicroscopy). Attached neutrophils spread over hyphal surfaces both within and beneath the endothelial cells; neutrophil recruitment to initial sites of leukocyte–Candida–endothelial cell interactions continued throughout the first 60 minutes of observation. Neutrophil spreading and stasis were observed only along Candida hyphae and at sites of Candida–endothelial cell interactions. These events resulted in 58.0% killing of Candida at 2 hours and subsequent clearance of Candida from endothelial cell monolayers, as determined by microcolony counts and morphological observation. On introduction of additional neutrophils to yield higher ratios of neutrophils to endothelial cells (10 neutrophils:1 endothelial cell), neutrophil migration toward hyphal elements continued. Despite retraction or displacement of occasional endothelial cells by invading Candida and neutrophils, most endothelial cells remained intact, viable, and motile as verified both by morphological observations and measurement of ^{51}Cr release from radiolabeled monolayers. From these studies, we conclude that neutrophils are capable of killing Candida hyphae selectively within human vascular endothelial cell monolayers and may have protective rather than detrimental effects on endothelial cell integrity.

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DISSEMINATED candidiasis is an increasingly important systemic opportunistic infection that is usually associated with widespread hematogenous seeding of multiple organs. It occurs most often in conjunction with neutropenia or neutrophil dysfunction.[1] *Candida albicans*, the species most commonly associated with dissemination, forms budding yeasts or blastospores that enlarge into pseudohyphae or germinate to form hyphae during the process of tissue invasion. Blastospores can be ingested or killed by circulating phagocytic cells,[2-5] and both neutrophils or monocytes can kill either pseudohyphae or hyphae, even though the latter forms are sufficiently large to preclude complete engulfment.[6-8]

Our previous studies have shown that Candida blastospores and hyphae can adhere to, be internalized by, and germinate within vascular endothelial cells. Because endothelial cells have no apparent fungicidal activity, the organisms can transgress through them to damage surrounding tissues.[9-11] This endothelial cell uptake is even more likely to occur if neutrophils are either absent or dysfunctional and fail to clear blood-borne Candida, or if a large inoculum of organisms enters the circulation. Klotz and colleagues[12] suggested that such uptake and growth of Candida leads to lytic endothelial cell damage and thereby may contribute to the pathogenesis of disseminated candidiasis. Vascular invasion and subsequent infarction is a hallmark of the histopathology of opportunistic mycoses.[13]

Because Candida can be internalized by endothelial cells and germinate within them, it is likely advantageous for the host to clear developing intraluminal hyphae prior to internalization or endothelial cell invasion with subsequent thrombotic infarction of tissues. Neutrophils are the most likely candidates to mediate such intraluminal hyphal clearance.[1-8] Of considerable importance is the demonstration that neutrophils exposed to a variety of soluble activators can adhere to, damage, and lyse vascular endothelial cells.[14-20] These studies have suggested a putative role for neutrophils in the pathogenesis of atherosclerosis, pulmonary vascular injury, or vasculitis.

The present studies address the effects of neutrophils on endothelium under conditions in which the neutrophil is activated by, and targeted against, an angioinvasive organism intimately associated with neighboring endothelial cells, and known to stimulate neutrophil degranulation and release of toxic oxygen species. These studies demonstrate that neutrophils selectively kill Candida hyphae within human vascular endothelial cell monolayers, and, in the process, may have protective rather than detrimental effects on endothelial cell integrity.

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MATERIALS AND METHODS

Organisms. *C albicans* (ATCC strain no. 36082), a clinical isolate, was grown in yeast nitrogen base (YNB) broth (Difco Laboratories, Detroit) supplemented with 1% dextrose (J.T. Baker Chemical, Phillipsburg, NJ) and 0.15% L-asparagine (Sigma Chemical, St Louis). Organisms were inoculated from modified Sabouraud's agar slants (containing chloramphenicol) into the YNB broth for overnight culture on a rotating drum at room temperature. Candida blastospores were washed three times, gently sonicated to produce singlets (3 seconds, setting no. 4; Sonicator model 350; Branson Sonic Power, Danbury, CT), standardized in medium 199 (M199) to 5×10^8 blastospores/mL, and added to endothelial cell monolayers at a final concentration of 5×10^5 blastospores/cm^2 (4.5×10^6/dish based on 9.0 cm^2 area for cell culture).

Endothelial cell culture. Human umbilical vein endothelial cells were obtained by the method of Jaffe and colleagues,[9,21] grown to confluence on 35-mm tissue culture dishes coated with extracellular matrix, and maintained in M199 supplemented with 2 mmol/L of L-glutamine, 20% fetal calf serum (FCS) (media and supplements from GIBCO, Grand Island, NY), and 250 ng/mL of fibroblast growth factor (provided by D. Gospodarowicz, University of California, San Francisco) as previously described.[9-22] Endothelial cells were enumerated by counting following trypsinization of confluent monolayers. Dishes contained 2.1×10^6 (± 0.3 SD) endothelial cells.

Leukocyte preparation. Leukocytes were separated from heparinized human blood by dextran sedimentation followed by NH_4Cl lysis of contaminating erythrocytes.[23] Leukocytes were washed, resuspended in M199 with 10% autologous serum, and standardized to 1×10^7 viable neutrophils per milliliter (as determined by trypan-blue exclusion and Wright's staining). Preparations were composed of 64% to 78% neutrophils, 2% to 8% monocytes, 1% to 3% eosinophils, and 20% to 31% lymphocytes.

Incubation of leukocytes with infected monolayers. At timed intervals, leukocytes were added to Candida-inoculated endothelial monolayers at ratios of neutrophils to blastospores ranging from 1:1 to 50:1, mixed gently, and allowed to settle onto the endothelial cell monolayer.

^{51}Cr Labeling and Candida inoculation of endothelial cell monolayers. Confluent endothelial cell monolayers were washed twice with 2 mL of M199, covered with 1 mL of M199 supplemented with 25 mmol/L of Hepes (pH 7.4), and 30 μCi of ^{51}Cr (added as Na_2CrO_4). Monolayers were incubated for 2 hours at 37°C in a 5% CO_2 atmosphere; the radiolabeled cells were washed extensively with M199 to remove unincorporated isotope. Following incubations (in triplicate) with or without addition of Candida or leukocytes, the endothelial cell cultures were washed three times, and the washes were pooled for γ counting. Total incorporated ^{51}Cr was determined by lysis of monolayers with 1 mL of 1 N NaOH. In preliminary experiments, neither endothelial cells, leukocytes, nor Candida reincorporated ^{51}Cr in lysates of radiolabeled endothelial cells. Data are expressed as percentage of specific ^{51}Cr release according to the formula: Percentage of specific release equals stimulated release minus spontaneous release/divided by total incorporated minus spontaneous release times 100.

Time-lapse videomicroscopy. Cultures were observed with an Olympus (New Hyde Park, NY) IMT-2 inverted-phase contrast microscope in a 37°C thermostatted enclosure (fabricated with the assistance of Donald Bottaro). Continuous recordings were made at $\frac{1}{12}$ to $\frac{1}{36}$ real time on Maxell (Secaucus, NJ) Professional-Industrial Epitaxial HGX-Pro-T120 videotapes using a Panasonic CCTV camera (model WV1460) interfaced to a Panasonic time-lapse video cassette recorder (model AG 6010), and viewed on a model TR 930 Panasonic video monitor (Matsushita Communications Industrial, Tokyo). At selected times, photographs were taken on an Olympus OM-G camera positioned at an independent microscope port.

Electron microscopy. Cells were fixed in situ by the addition of phosphate-buffered glutaraldehyde/formaldehyde (final concentrations 1% and 4%, respectively), as previously described.[8] For transmission microscopy, cells were sequentially stained with 1% aqueous osmium tetroxide for 60 minutes and 0.1% aqueous uranyl acetate for 30 minutes and dehydrated in graded ethanols containing 0.1% toluidine blue. One micrometer-thick sections were cut en-face or perpendicular to the plane of the endothelial monolayer. Ultrathin sections were cut with diamond knives, stained with lead citrate and uranyl acetate, and examined with a Philips EM300 at 80 kV (Philips Electronic Instruments, Mahwah, NJ).

Determination of fungal viability. As in our previous studies, both microculture and vital staining methods were used to determine fungal viability following the interaction of fungi with leukocytes.[24,25]

For the microculture method, endothelial cell monolayers were grown in multiwell plastic tissue culture dishes (Corning Lab-ware, Corning, NY) and inoculated with Candida as described above. To control those wells in which leukocytes were incubated with endothelium and Candida, at the conclusion of incubations corresponding numbers of leukocytes were added to the leukocyte-free wells (this step was performed to account for any effects that products released from leukocytes might have on the organisms during the process of quantitative culturing). Then the monolayers in both the wells that contained leukocytes during incubation and those that did not contain leukocytes during the incubation (but had them added at the end of the incubation) were immediately washed to remove the nonadherent Candida and leukocytes. All the wash fluid supernatants were collected in corresponding glass test tubes for quantitative cultures. After leukocyte lysis (described below), the Candida remaining in these test tubes were centrifuged and the supernatant was discarded. The organisms were then washed in distilled water, resuspended, and aliquots were removed for quantitative pour-plating in Sabouraud's agar.

For lysis of the leukocytes remaining adherent to the monolayers and those in the corresponding supernatant washes, 0.25% sodium deoxycholate was used. The deoxycholate treatment lysed neutrophils completely, but incomplete lysis of the endothelial cells resulted. Therefore, hyphae remained adherent to bottoms of the plastic chambers on the surface of the endothelial cell remnants. To quantify those hyphae remaining, 100 μL of Sabouraud's agar were overlaid over the chamber bottoms and incubated at 37°C for 4 to 6 hours. The chamber bottoms were then examined by inverted-phase microscopy for determination of both the remaining number and the viability of the organisms remaining.

For the vital staining technique, the yellow tetrazolium dye 3-[4,5-dimethylthiazol --2yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) was used. It served as both a qualitative marker of fungal and endothelial cell viability (by direct visualization) and a method for quantitation of fungal cell viability by extraction of precipitated intracellular MTT-formazan, as previously described.[24,25]

For quantifying fungal cell viability, endothelial cell monolayers were grown in Lab-tek tissue culture chambers (Miles Scientific, Naperville, IL), inoculated with Candida and leukocytes, incubated briefly, and washed with phosphate-buffered saline (PBS) to remove nonadherent cells. The dishes then were incubated for 3 hours in 0.5 mg/mL of MTT in Dulbecco's PBS. At the end of the incubation, they were washed twice with buffer saline. Stained fungi, leukocytes, and endothelial cells contained clearly visible, 1- to 2-μm purple crystals of MTT-formazan. To quantify fungal viability, leukocytes and endothelial cells were lysed in deoxycholate, the released MTT-formazan was removed by washing with distilled water, and the wash supernatants, containing some suspended Candida, were pooled in centrifuge tubes to separate the Candida. The MTT-formazan was extracted from hyphae in the centrifuged pellets and from those remaining adherent on slides, by incubation for 5 minutes in 0.4 mL of acidified isopropyl alcohol (0.04 mol/L of HCl in isopropanol). Controls using our standard procedure[25] verified that deoxycholate lysis caused no release of MTT-formazan from the Candida. From each culture well and cell pellet, 300 μL of extract containing the MTT-formazan was then removed and placed in a flat-bottomed microwell of a multiwell plate. Absorption at 570 nm of each well was determined using an Artek manual enzyme-linked immunoassay (ELISA) reader (Dynatech, Alexandria, VA) equipped with a 570-nm filter, and calibrated to 0 absorbance on wells containing extracts from chambers that initially contained only medium and MTT. Total viable organisms remaining in experimental wells could then be determined by calculating the sum of values obtained from extracts of adherent cells and cell pellets obtained from the centrifugation of the wash supernatants.

Using these methods, the percentage of the Candida inoculum that adhered to the endothelium could be determined as could the percentage of those Candida that adhered to the endothelium and were subsequently killed by the leukocytes. The low starting inoculae permitted direct plating of samples from the pooled wash supernatants, resulted in minimal clumping and, in leukocyte-free controls, resulted in recovery of >90% of the original blastospore inoculum as colony-forming units (CFUs). The total number of CFUs (viable organisms) remaining under various experimental conditions could thus be determined from the sum of viable counts in the wells added to the quantitative counts of the wash supernatants.

RESULTS

As in our previous experiments,[9] Candida blastospores adhered to, were ingested by, and subsequently germinated within monolayers of human umbilical vein endothelial cells. Endothelial cell monolayers remained intact within this initial 2-hour period. Those endothelial cells in contact with germinating Candida remained viable, as determined by maintenance of cell motility documented during continuous time-lapse video taping.

Time-lapse videotaping was then used to visualize sequential events following addition of neutrophils after 2 hours of Candida germination. As shown in Fig. 1A, neutrophils first settled and then migrated toward hyphal tips protruding from endothelial cells in the monolayer. After attaching at the junction between protruding hyphae and endothelial cells, the neutrophils penetrated through the monolayer along the hyphae (Fig 1C). Transverse sections (not shown) indicated that neutrophils often extended through, and even under, the endothelial cell layer along the course of the hyphae. After attachment of the initial neutrophils at hyphal–endothelial cell junctions, additional neutrophils migrated selectively to these original neutrophil attachment sites (Fig 1A and B). No neutrophil attachment was

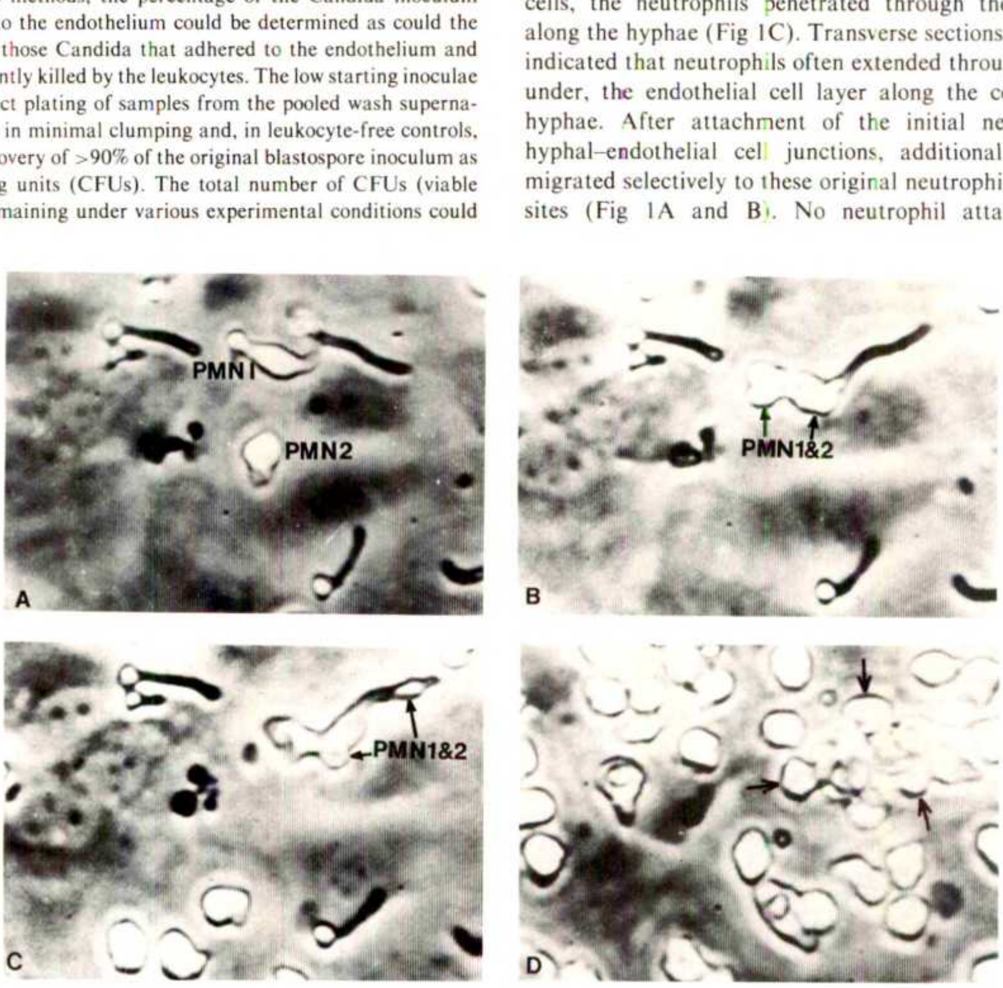

Fig 1. Videomicroscopy of interactions of neutrophils (polymorphonuclear leukocytes, PMNs) and *Candida albicans* hyphae emerging from endothelial cell (E) monolayers (original magnification × 1,700; current magnification × 663). (A) Neutrophil (PMN1) has attached to the end of a Candida hyphus that was above the plane of the monolayer. A second neutrophil (PMN2) is migrating toward the PMN1–hyphal interaction (17 minutes). (B) PMN2 has joined PMN1 and is beginning to spread along the length of the hyphus (20.5 minutes). (C) Progressive spreading of neutrophils to cover the entire hyphal surface within monolayer (32 minutes). (D) Introduction of additional neutrophils leads to their migration and accumulation (arrows) at sites of initial interactions between neutrophils and hyphae (64 minutes).

observed at sites other than those of hyphal protrusion through the monolayer.

During the next 30 minutes, neutrophils spread over the hyphae within the monolayer to cover hyphal surfaces (Fig 1C). Those endothelial cells in immediate proximity to the Candida-adherent neutrophils were morphologically normal and retained their motility. After the first hour of Candida–neutrophil interaction, additional neutrophils (20 per hyphae) were added, approximating ratios of neutrophils to endothelial cells that resulted in endothelial cell damage in prior studies.[14,15,17,20] The vessels were shaken to facilitate even distribution of these added neutrophils. The neutrophils sedimented onto the monolayer and selectively migrated to join the original neutrophils that already surrounded the hyphae (Fig 1D). The remainder of the endothelial cell monolayer, those cells not in contact with the hyphae and attached neutrophils, appeared normal.

The local effects of neutrophils on endothelial cells in proximity to Candida hyphae were determined by transmission electron microscopy from fixed smears obtained at timed intervals during time-lapse videotaping experiments. In the process of migrating along hyphae into the monolayer, some neutrophils displaced endothelial cells by penetration under the monolayer (not shown). When high ratios of neutrophils to hyphae (50:1) were added, examination by phase-contrast microscopy revealed disruption of some areas of the endothelial cell monolayer immediately adjacent to the large clusters of neutrophils that surrounded the hyphae (Fig 2).

Overall, addition of neutrophils decreased monolayer damage and endothelial cell cytolysis caused by Candida, as determined by release of ^{51}Cr from radiolabeled endothelium measured at timed intervals in three separate experiments. In the absence of neutrophils, progressively germinating Candida significantly damaged the monolayers by 4 hours of incubation and caused most of the incorporated ^{51}Cr to be released by 6 hours (Fig 3). In contrast, as shown in Fig 3, neutrophils caused only a minimal release of ^{51}Cr when applied to uninfected monolayers for 4 hours and did not significantly increase ^{51}Cr release from the infected endothelial cell monolayers despite the avid interaction of neutrophils with hyphae in infected monolayers. Interacting neutrophils and hyphae did not reincorporate ^{51}Cr after initial release during control experiments using supernatants from lysed endothelial cells. Specifically, supernatants from lysed endothelial cells were incubated at 37°C for 1 hour with 10^7 Candida alone, 5×10^7 neutrophils alone, and with Candida and neutrophils combined. The preparations were centrifuged, and the supernatants and pellets were counted. In each instance, cell reuptake of previously released ^{51}Cr was insignificant (0.0% to 2.8%). Thus, neutrophils significantly reduced Candida-induced ^{51}Cr release from radiolabeled endothelial cells ($P < .001$ by two-tailed Student's *t* test for paired samples).

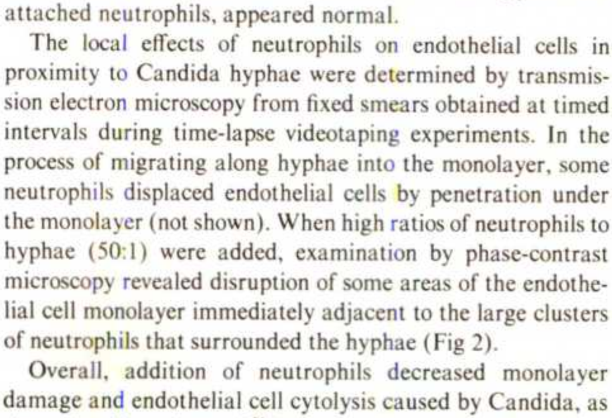

Fig 2. Limited dislodgement and retraction of occasional endothelial cells, leaving focal gaps in the area of monolayer (arrow) immediately adjacent to a large cluster of neutrophils surrounding *Candida albicans* hypha, following addition of leukocytes at a ratio of 10 neutrophils per endothelial cell (original magnification × 420; current magnification × 206).

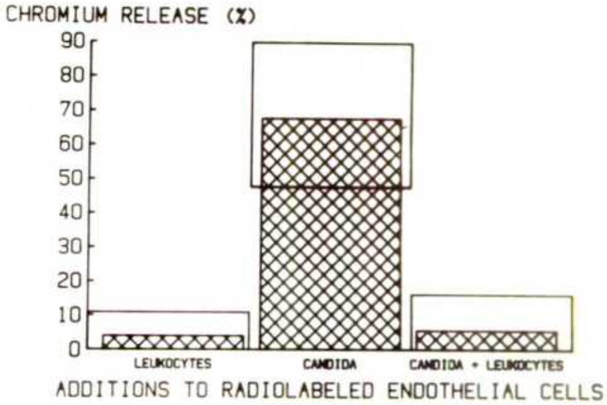

Fig 3. Effects of hyphae and/or neutrophils on cytolysis of endothelial cells as determined by ^{51}Cr release. Hatched bars represent mean (three separate experiments, each repeated in triplicate) specific release of ^{51}Cr from radiolabeled endothelial cell monolayers exposed, respectively, either to neutrophils (five to each endothelial cell) for 4 hours (leukocytes) or *C albicans* blastospores (one to each endothelial cell), and subsequently washed to remove nonadherent fungi and then incubated to support progressive germination and hyphal growth within monolayers for 6 hours (Candida); or, after initial germination of hyphae in monolayers for 2 hours, five neutrophils were added to each Candida, and incubations were continued for an additional 4 hours (Candida + leukocytes). Open rectangles superimposed on hatched bars denote 95% confidence limits. Hyphal growth significantly increased endothelial cell cytolysis, but this increase was reversed by the addition of neutrophils ($P < .001$).

Endothelial cells by themselves had no apparent candidacidal activity. Within endothelial cell monolayers, the interaction of neutrophils with hyphae led to depletion of granules in the areas immediately adjacent to the neutrophil–hyphal interface, as observed microscopically (Fig 4). Comparable levels of candidacidal activity to those observed in simultaneous incubations of Candida hyphae with neutrophils in the absence of endothelial cells was seen (58.0% killing after 2 hours of incubation with neutrophils in endothelial cell monolayers *v* 49.4% killing in plastic dishes in the absence of endothelial cells). The fungicidal activity was determined by agar overlay following 2-hour incubations and subsequent quantitative counts of microcolonies formed on the plates and added to quantitative microcolony counts of Candida

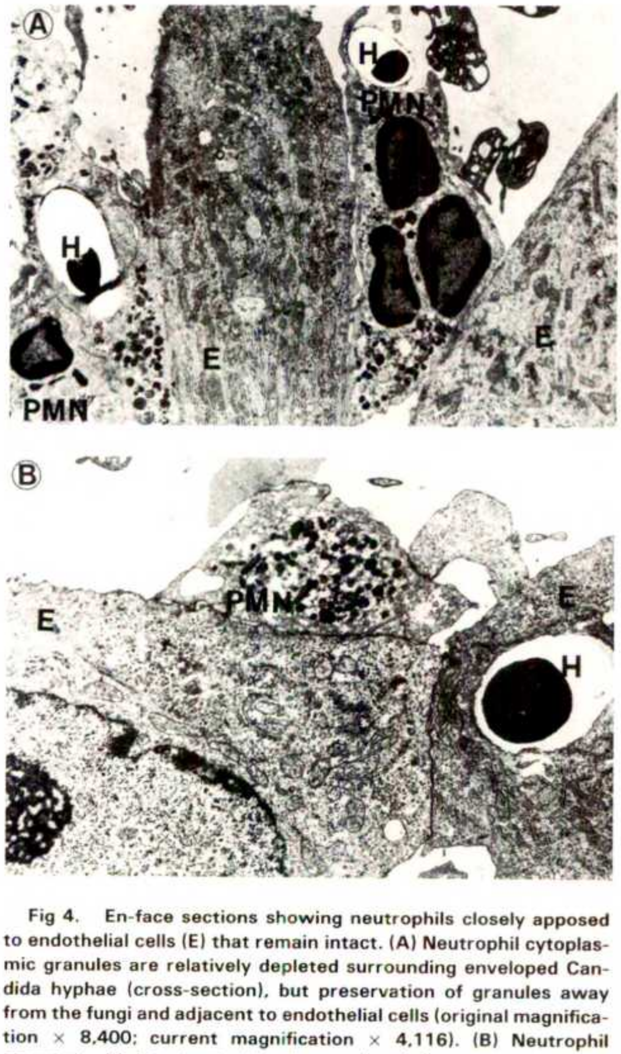

Fig 4. En-face sections showing neutrophils closely apposed to endothelial cells (E) that remain intact. (A) Neutrophil cytoplasmic granules are relatively depleted surrounding enveloped Candida hyphae (cross-section), but preservation of granules away from the fungi and adjacent to endothelial cells (original magnification × 8,400; current magnification × 4,116). (B) Neutrophil segment with intact cytoplasmic granules during penetration into monolayer, where adjacent endothelial cells remain intact (original magnification × 21,573; current magnification × 10,571.

within supernatants of the cultures. Results obtained using the MTT dye assay for quantitative confirmation of hyphal viability substantiated these findings. Verification that reduction in colonies was not due to clumping was obtained through direct observation by phase-contrast microscopy throughout the period of incubation and microcolony formation. Observations made during the time-lapse studies suggested that neutrophils removed (and on occasion appeared actively to pull out) hyphae from the endothelial cell layers. These observations were confirmed by studies performed at timed intervals. When monolayers were washed free of adherent neutrophils, after 90 minutes of incubation, a 38.2% reduction occurred in the number of Candida hyphae, although most of the endothelial cell monolayer remained intact. In contrast, when no neutrophils were added to the hyphae growing within the endothelial cells, massive destruction of the monolayers occurred after 6.5 hours of incubation (Fig 5A), and monolayers were completely destroyed by hyphal overgrowth after 18 hours. At these later intervals, when neutrophils were added to endothelial cells that contained progressively growing Candida, the monolayers were largely preserved and remaining hyphae were dramatically reduced in number. As shown in Fig 5C, washing endothelial cells free of adherent neutrophils after 4.5 hours of incubation (6.5 hours of Candida growth within the endothelium) revealed monolayers that were far better preserved than those incubated without neutrophils. Endothelial cells retained viability as judged by trypan blue dye exclusion as well as the more sensitive index of retained mobility determined by time-lapse videotaping.

Staining with the tetrazolium dye MTT also was used as an index of viability for both hyphae and endothelial cells. Both of these eucaryotic cells contain mitochondrial dehydrogenases that reduce the dye to visible, intracellular precipitates of its formazan derivative. After 18 hours of incubation, once almost all the neutrophils have been removed by washing from the endothelial cell monolayers, MTT staining revealed that only occasional viable hyphae, all very small in size, remained, whereas many live endothelial cells were present. Time-lapse videotaping demonstrated that motility was preserved in these stained, residual endothelial cells, confirming their viability. Consistent with these findings was the observation that when low ratios of neutrophils to hyphae (1 to 5:1) were used, though neutrophils attacking hyphae appeared to be closely apposed to endothelial cells, the adjacent endothelial cells remained morphologically normal (Fig 4A and B). Moreover, although neutrophils appeared to be depleted of granules in areas in immediate proximity to sites of their hyphal attachment, large numbers of intact granules remained within the cytoplasms of neutrophils immediately adjacent to those endothelial cell surfaces far removed from Candida.

DISCUSSION

As in previous studies,[9] *C albicans* was internalized in vitro by human vascular endothelial cells, apparently without diminishing the ability of the organism to germinate. In the previous study, internalized Candida were restricted to endothelial phagocytic vacuoles, as observed by transmission electron microscopy. The longer incubations used in the present study presumably allowed progressive Candida germination, ending in endothelial cell lysis. When neutrophils were added, they were attracted to surface-adherent Candida and to sites where germinated hyphae protruded through the endothelial monolayer. Attached neutrophils then penetrated the endothelial monolayer along the course of hyphal filaments, spread on hyphae, killed them, and cleared them from the monolayer. In earlier studies, we showed that the candidacidal activity of neutrophils depends largely on activation of the respiratory burst and release of toxic oxygen species.[6-8] Limited loss of endothelial cells from monolayers occurred, but only at sites of leukocyte–Candida interactions. Although diffusely infected by Candida, endothelial monolayers were largely preserved by the addition of leukocytes, as determined both by ^{51}Cr release and morphological criteria. In contrast, when neutrophils were absent,

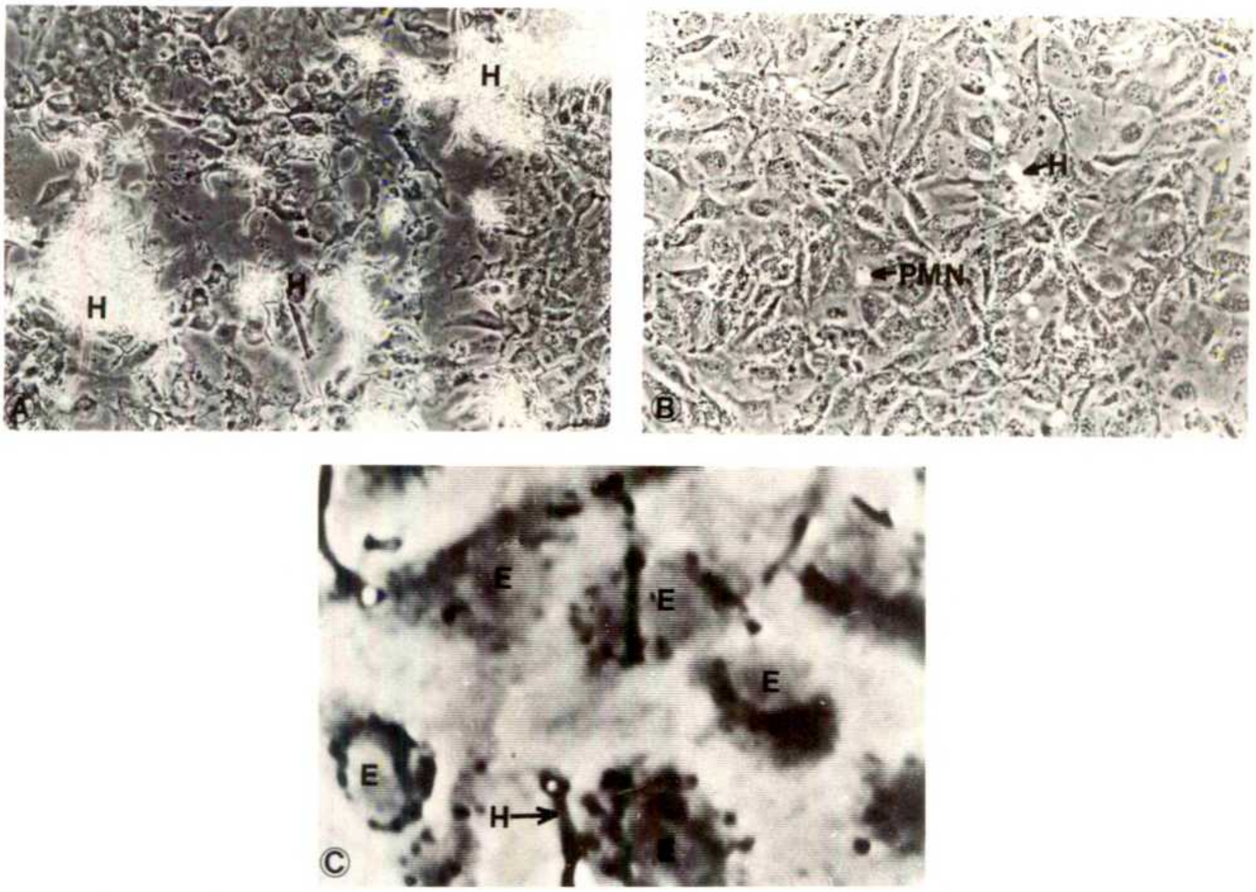

Fig 5. Effects of progressive hyphal elongation on morphological integrity of endothelial cell monolayer. (A) Extensive destruction of monolayer as evidenced by endothelial cell loss and retraction after 6 hours of hyphal growth to form large individual filaments, clusters, and microcolonies (original magnification × 275; current magnification × 135). (B) Hyphae were germinated on endothelial cell monolayer for 2 hours prior to the addition of leukocytes; monolayers were incubated for an additional 4 hours (6 hours total), and washed to remove nonadherent neutrophils. Overall, monolayer is preserved, with only rare remaining hyphal filaments (original magnification × 275; current magnification × 135). (C) Parallel preparation (performed simultaneously with [B] with hyphae and neutrophils incubated on endothelial cell monolayer for 18 hours, and stained with 3-[4,5-dimethylthiazol—2yl]-2,5-dyphenyltetrazolium bromide (MTT), showing preservation of viable endothelial cells and only occasional, short hyphal filaments containing precipitated, dark crystals of MTT-formazan indicating cell viability (original magnification × 1,300; current magnification × 637).

Candida hyphae grew progressively and completely destroyed endothelial cell monolayers.

These findings differ in certain critical respects from previous studies that focused on aspects of the neutrophil–endothelial interaction as potential mechanisms underlying intravascular injury occurring in adult respiratory distress syndrome and systemic vasculitis or in the development of atherosclerosis.[12-18] Depending on experimental conditions, neutrophils may cause either nonlytic retraction and detachment of endothelial cells[14,16] or significant endothelial cell lysis.[15,17-19] Other researchers have suggested that close apposition of cells is required for neutrophil-mediated endothelial damage[15,16] and that activation of the neutrophil respiratory burst[14-19] is necessary. Thus, endothelial cell damage has been produced by normal and myeloperoxidase-deficient, but not by chronic granulomatous disease neutrophils,[17] and has been inhibited by blockade of the respiratory burst by dapsone,[18] as well as by catalase,[15,17,19] and scavengers of toxic oxygen species.[14-19] Endothelial cell damage has been induced by a variety of cell-free oxidant-generating systems, including the myeloperoxidase-hydrogen peroxide-halide system,[17-19] by hydrogen peroxide generation from glucose plus glucose oxidase,[17] or by superoxide generation from xanthine plus xanthine oxidase.[15,18,19] In other studies, release of neutral proteases has been implicated as a nonoxidative mechanism whereby neutrophils mediate detachment, but not lysis, of cultured endothelial cells.

In studies demonstrating endothelial cell lysis, neutrophils have generally been stimulated by soluble mediators (including phorbol myristate acetate (PMA),[16-19] C5a[15], or the chemotactic peptide formyl-methionyl-leucyl-phenylalanine[14]), or by opsonized zymosan,[20] under conditions likely to lead to global activation of the neutrophil. In our studies, we intentionally avoided using mediators that would result in global activation; instead we focused on an activation mechanism that was likely to be physiologic within the context of the interaction of neutrophils, endothelium, and the pathogenic microorganism, Candida. Neutrophils are capable of selectivity in the release of microbicidal products and mediation of target cell damage when stimulated by particulate substrates. For example, neutrophils adherent to nonphagocytizable surfaces release oxidants focally, only at sites of attachment.[26] We previously showed that Candida hyphae are killed only by closely adherent neutrophils, primarily by oxidative mechanisms.[6-8,27] Hyphal killing is likely the result of a focal interaction confined specifically to those areas of contiguity between the target and the phagocyte.

Several recent observations indicate that endothelial cells participate actively in neutrophil adherence and generation of inflammatory products at the vessel wall. Under appropriate conditions, endothelial cell monolayers possess antiadhesive properties that are protective against stimulated neutrophils.[28,29] Alternatively, endothelial cells may respond with elaboration of soluble and membrane-bound proinflammatory signals, including platelet-activating factor[30] and a recently described class of endothelial-dependent leukocyte-dependent adhesion molecules[31,32] capable of augmenting neutrophil adherence and activation. The possibility that Candida or other microorganisms elicit local changes in endothelial cell metabolism that influence subsequent neutrophil activation warrants further investigation.

In summary, these studies extend our earlier observations on pathogenetic mechanisms invoked by angioinvasive fungi. In the present study, unimpeded hyphal elongation was associated with extensive damage to endothelial cells. Activated leukocytes conferred protection to infected endothelial monolayers despite demonstrated fungicidal activity, ultrastructural evidence of neutrophil degranulation, and presumed activation of the neutrophil respiratory burst. Whereas prior studies have shown extensive lytic and nonlytic endothelial damage by activated neutrophils, the data we present suggest that, under appropriate conditions, "targeted" neutrophils can effect microbial killing with minimal and focally limited damage to neighboring endothelium.

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Posttransfusion Purpura Associated With an Autoantibody Directed Against a Previously Undefined Platelet Antigen

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Although alloantibody against the PLA^1 platelet antigen is usually found in patients with posttransfusion purpura (PTP), the mechanism of destruction of the patient's own PLA^1-negative platelets is unexplained. We used a sensitive immunoblot technique to detect antiplatelet antibodies in a patient with classic PTP. The patient's acute-phase serum contained antibodies against three proteins present in control (PLA^1-positive) platelets: an antibody that bound to a previously unrecognized platelet protein of mol wt 120,000 [glycoprotein (GP) 120], antibodies that bound to PLA^1 (mol wt 90,000), and an epitope of GP IIb (mol wt 140,000). The antibodies against PLA^1 and GP IIb did not react with the patient's own PLA^1-negative platelets, control PLA^1-negative platelets, or thrombasthenic platelets. In contrast, the antibody against GP 120 recognized this protein in all three platelet preparations, but not in Bernard-Soulier or Lek^a (Bak^a)-negative platelets. Antibody against GP 120 was not detected in the patient's recovery serum, although the antibodies against PLA^1 and GP IIb persisted. F(ab)$_2$ prepared from the patient's acute-phase serum also bound to GP 120. These results suggest that in PTP, transient autoantibody production may be responsible for autologous (PLA^1-negative) platelet destruction. In addition, alloantibodies against more than one platelet alloantigen may be found in this disease. The nature of the GP 120 autoantigen and the GP IIb-related alloantigen defined by our patient's serum remains to be determined.
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POSTTRANSFUSION purpura (PTP), first described >25 years ago,[1-3] is considered "an enigma of alloimmunization."[4] PTP is an acute bleeding disorder characterized by the onset of thrombocytopenia ~1 week following blood transfusion.[4-9] The syndrome occurs predominantly in patients who lack the platelet-specific alloantigen, PLA^1, and who have been sensitized by prior transfusion or pregnancy.[5] Although antibodies against PLA^1 are usually present in patient serum, the mechanism of destruction of the patient's own PLA^1-negative platelets remains unexplained. Recently, antibody against another platelet-specific antigen, Lek^a, was detected in the serum of a patient with PTP.[10,11] As in many cases of this disease, however, the patient died before autologous platelets could be obtained for study, so that the relationship between production of anti-Lek^a antibody and destruction of the patient's own platelets remains uncertain. Both PLA^1 and Lek^a are deficient in thrombasthenic platelets that lack the platelet glycoprotein IIb/IIIa (GP IIb/IIIa) complex.[10] The PLA^1 epitope has been localized to GP IIIa,[12] whereas Lek^a appears to be an epitope of GP IIb.[11] Lek^a now appears to be identical to the Bak^a alloantigen.[13]

Prompt recognition of PTP and treatment with large-volume plasmapheresis has led to improved survival in this disease.[14,15] These factors allowed us to perform detailed antibody binding studies in a patient who recovered from severe thrombocytopenia associated with a classic case of PTP.

MATERIALS AND METHODS

Case report. A 71-year-old multiparous woman underwent transabdominal hysterectomy because of uterine fibroids. She had no history of prior transfusion, bleeding, or thrombocytopenia, and her preoperative platelet count was 220,000/μL. During surgery, 2 U of packed RBCs were transfused, and postoperatively the patient's hematocrit and platelet count were normal. Eight days after surgery, however, the patient developed petechiae and mucosal bleeding, and her platelet count was 6,000/μL. There was no clinical or laboratory evidence of infection, hemolysis, or disseminated intravascular coagulation. The platelet-associated immunoglobulin level measured by a fluorescence-activated flow cytometric technique[16] was elevated. The patient was taking no medications.

PTP was suspected, and an 11-L plasmapheresis was performed over 4 days using total-volume replacement with fresh frozen plasma. On the third day, the patient's platelet count rose to 170,000/μL and remained in the normal range. No further bleeding occurred and the patient was discharged from the hospital. One month after recovery, her platelet-associated immunoglobulin level was normal. The patient's platelets were PLA^1-negative, as shown by an enzyme-linked immunoassay[17] performed at Irwin Memorial Blood Bank, San Francisco, using high-titer anti-PLA^1 antiserum. The patient's acute-phase serum obtained prior to plasmapheresis contained significant anti-PLA^1 antibody, as demonstrated by indirect platelet immunofluorescence and ^{51}Cr release cytotoxicity (kindly performed by Dr Richard Aster of the Blood Center of Southeastern Wisconsin, Milwaukee). Antibodies against PLE^1 and Bak^a (Lek^a) were not detected by these techniques. The patient's HLA type was A2 A3 B8 B35, as determined by the standard lymphocytotoxicity assay.[18]

Platelet preparations. Platelets were prepared from the following donors: a PLA^1-positive control, a PLA^1-negative control, two patients with Glanzmann's thrombasthenia known to have <10% GP IIb/IIIa,[19] and two patients with Bernard-Soulier syndrome. The Bernard-Soulier platelets were generously provided by Dr Margaret Johnson of the Medical Center of Delaware, Newark. Autologous platelets were obtained from the patient 1 month after recovery. Platelets were also obtained from the patient's older son, who is

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PLA^1-positive, and from her younger son, who is PLA^1-negative. Lek^a (Bak^a)-negative platelets from two patients were the generous gift of Dr Alan Nurden of the Hôpital Lariboisière, Paris. All platelets were washed and solubilized in sodium dodecyl sulfate (SDS) sample buffer, as previously described.[16] For reduction of platelet proteins, 200 mmol/L of dithiothreitol (DTT) was added to the control platelet preparation prior to gel electrophoresis.

Neuraminidase treatment of control platelets was performed by a modification of the technique of McGregor and colleagues.[20] In brief, 10^9 washed platelets were suspended in 1 mL of phosphate-buffered saline (PBS) containing 5 mmol/L of EDTA. The platelets were incubated with 1 U of protease-free *Vibrio cholerae* neuraminidase (Calbiochem, San Diego) for 15 minutes at 37°C. They were then washed three times in PBS and solubilized in SDS sample buffer at the same concentration as untreated platelets (10^9/mL).

Antibodies and platelet eluates. Serum was obtained from the patient during the acute phase of her disease immediately prior to plasmapheresis. Serum was also obtained from the patient 1 month after recovery. F(ab)$_2$ immunoglobulin fragments were prepared from the patient's acute-phase serum by sequential protein A-Sepharose purification and pepsin digestion of IgG.[16] Rabbit antiserum against GP IIb/IIIa[19] was generously provided by Dr Rod McEver of the University of Texas Health Science Center, San Antonio, TX.

Affinity isolation of serum antibody was performed using a modification of the acid elution technique described by Falus and colleagues.[21] In brief, 5 × 10^9 washed platelets were fixed with 1% paraformaldehyde[16] and incubated for 1 hour with 500 μL of test serum diluted 1:1 in PBS. The platelets were washed three times in PBS and incubated with 0.5 mL of 0.1 mol/L of glycine, pH 2.5, for 5 minutes. The platelets were sedimented, and the supernatant eluate was neutralized with 1 mol/L of Tris, pH 8.0. The eluate was dialyzed against PBS and stored at −70°C until use.

Eluates were prepared using acute and convalescent patient sera, as well as control serum and high titer anti-PLA^1 antiserum. Binding of the eluted antibody to control or PLA^1-negative platelets was determined using the fluorescence-activated cell sorter (FACS) assay, as previously described.[16] Eluate binding on immunoblots was performed as described below.

Immunoblotting. Binding of serum antibody, platelet eluates or F(ab)$_2$ fragments to platelet membrane proteins was detected by an immunoblot technique, as previously described.[22] In brief, SDS-solubilized platelets were electrophoresed in SDS-5% polyacrylamide gels under nonreducing conditions. The platelet proteins were transferred to nitrocellulose membranes and incubated with sera, eluates or F(ab)$_2$ (5 mg/mL) diluted 1:50 in Tris-buffered saline (TBS). Antibody binding was detected using a biotin-avidin-peroxidase system.[21] For antibody binding to reduced platelet proteins, DTT-treated platelets were substituted for unreduced platelets prior to electrophoresis. In the blocking experiment, the nitrocellulose membrane was first incubated with a 1:2,000 dilution of normal rabbit serum or rabbit anti-GP IIb/IIIa for 1 hour. After being washed in TBS, the nitrocellulose was incubated with patient serum for 2 hours. Antibody binding was detected using biotinylated goat F(ab)$_2$ anti-human immunoglobulin and avidin-peroxidase. The anti-human immunoglobulin does not cross-react with rabbit antibody.

RESULTS

Antibody binding to various platelet preparations using the patient's acute-phase serum (lanes A through D) and convalescent serum (lanes E through H) is shown in Fig 1. The acute-phase serum contained antibodies against three distinct proteins in control PLA^1-positive platelets, shown in lane A: an antibody that bound to PLA^1 (mol wt 90,000), an antibody that bound to an epitope of GP IIb (mol wt 140,000), and a third antibody that bound to a protein of mol wt 120,000 (GP 120). The antibodies against PLA^1 and GP IIb did not react with the patient's own PLA^1-negative platelets, shown in lane B. In contrast, the antibody against GP 120 did react with those platelets. The anti-PLA^1 and anti-GP IIb antibodies failed to bind to control PLA^1-negative platelets, shown in lane C, or to thrombasthenic platelets, shown in lane D. The antibody against GP 120, however, bound to this antigen in both control PLA^1-negative platelets (lane C) and thrombasthenic platelets (lane D).

Binding of anti-platelet antibody in the patient's convalescent serum is shown in lanes E through H. As shown in lane E, antibodies that bound to PLA^1 and the GP IIb epitope were still present after the patient's recovery. Antibody against GP 120, however, was no longer detected in the convalescent serum. Similarly, antibody binding to GP 120 was no longer detected using the patient's autologous platelets (lane F), control PLA^1-negative platelets (lane G), or thrombasthenic platelets (lane H). As in lanes B through D, the antibodies against PLA^1 and GP IIb failed to bind to these platelets.

Figure 2 shows the results of immunoblotting using F(ab)$_2$ prepared from the patient's acute-phase serum. The F(ab)$_2$ bound to GP 120 in control PLA^1-positive platelets, shown in lane A, and in the patient's own platelets, shown in lane B, indicating autoantibody binding to the target antigen. F(ab)$_2$

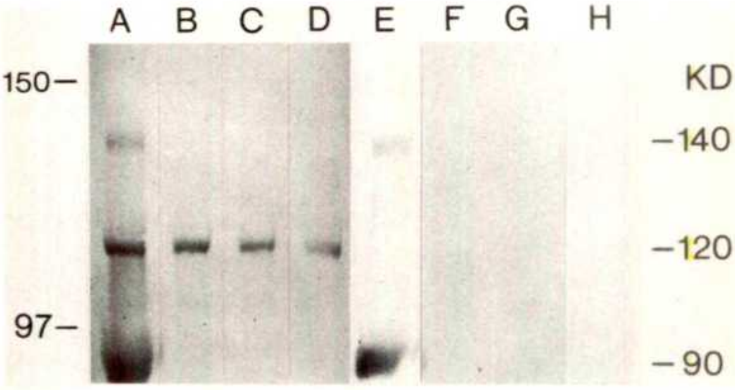

Fig 1. Binding of patient's serum antibody to platelet proteins. Solubilized platelet proteins were electrophoresed in sodium dodecyl sulfate (SDS) 5% polyacrylamide gels, transferred to nitrocellulose, and incubated with patient's acute-phase serum (lanes A through D) or convalescent serum (lanes E through H), as described in the Materials and Methods section. Lanes A and E, control PLA^1-positive platelets; lanes B and F, autologous (PLA^1-negative) platelets; lanes C and G, control PLA^1-negative platelets; lanes D and H, thrombasthenic platelets. Bands at mol wt 90,000 (PLA^1), mol wt 140,000 (glycoprotein [GP] IIb), and mol wt 120,000 (GP 120) are apparent in lane A. Text gives explanation of antibody binding in other lanes.

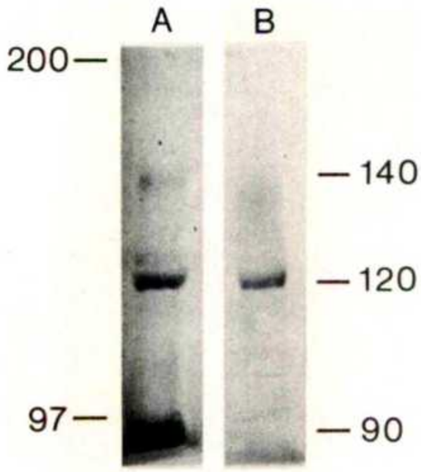

Fig 2. Binding of patient's F(ab)$_2$ to platelet proteins. Immunoblotting was performed as described in Fig 1, except that patient's F(ab)$_2$ at 5 mg/mL was substituted for serum. Lane A, control PLA^1-positive platelets; lane B, autologous PLA^1-negative platelets. Band of mol wt 120,000 was detected by F(ab)$_2$ in both lanes.

Table 1. Binding of Eluted Platelet Antibodies to Control or PLA^1-Negative Platelets

Source of Eluate	Binding to	
	Control Platelets	PLA^1-Negative Platelets
Control serum	Negative	Negative
Acute patient serum	Positive	Positive
Convalescent patient serum	Positive	Negative
Anti-PLA^1 antiserum	Positive	Negative

Serum samples were incubated with paraformaldehyde-fixed control platelets. Antibody elution was performed using acid-glycine. Eluates were then incubated with control or PLA^1-negative platelets. Antibody binding was detected using FACS. Details are described in the Materials and Methods section.

binding to PLA^1 and GP IIb was also seen in control platelets (lane A), although the intensity of binding was less than with whole serum. Serum antibody and F(ab)$_2$ failed to bind to RBCs, granulocytes, lymphocytes, or monocytes (data not shown).

Figure 3 shows the results of acute-phase serum antibody binding to other platelet preparations. Binding of antibody to Lek^a (Bak^a)-negative platelets is shown in lanes B and C. Although antibody binding to PLA^1 and GP IIb was seen in these platelets, antibody binding to GP 120 was not detected. Thus, Lek^a (Bak^a)-negative platelets appear to be deficient in the GP 120 antibody binding site. Binding of the patient's antibody to her two sons' platelets is shown in lanes D and E. The older son's PLA^1-positive platelets bound PLA^1 antibody, as shown in lane D. The younger son's platelets are deficient in PLA^1, however, and failed to bind anti-PLA^1 antibody. Antibody binding to GP 120 was observed in both platelet preparations. Neither son's platelets reacted with the anti-GP IIb antibody, however, indicating that this epitope of GP IIb is missing on both children's platelets. Antibody binding to a second thrombasthenic patient's platelets is shown in lane F. These platelets also showed antibody binding to GP 120 but not to PLA^1 or GP IIb. In contrast, serum antibody binding to PLA^1 and GP IIb was seen in Bernard-Soulier platelets, shown in lane G. Binding to GP 120 was not detected in these platelets. Thus, Bernard-Soulier platelets, like Lek^a (Bak^a)-negative platelets, appear to lack either GP 120 or the epitope that reacts with the anti-GP 120 autoantibody.

Table 1 shows the results of FACS analysis of binding of platelet antibody eluates to intact platelets. The eluates made from the patient's acute-phase serum bound to control platelets and PLA^1-negative platelets, indicating the presence of antibody against platelet determinants in addition to PLA^1. The convalescent serum eluate also reacted with control platelets, but failed to bind to the PLA^1-negative platelets. This finding is consistent with the disappearance of non-PLA^1 autoantibody in the recovery serum. It also demonstrates antibody binding on the surface of intact platelets fixed with paraformaldehyde, confirming that GP 120 is a membrane antigen.

Figure 4 shows the results of immunoblotting using the platelet eluates described in Table 1. The antibody eluate prepared from acute-phase serum bound to three epitopes on control platelets (lane A) but only to GP 120 on PLA^1 negative platelets (lane B). The antibody eluate from convalescent serum bound to GP IIb and PLA^1 (but not to GP 120) on control platelets (lane C). This eluate failed to bind to PLA^1-negative platelets (lane D). Thus, the results of immunoblotting using platelet eluates were identical to the serum antibody findings shown in Fig 1.

Figure 5 shows the results of three additional experiments that further characterize the target platelet antigens. Bind-

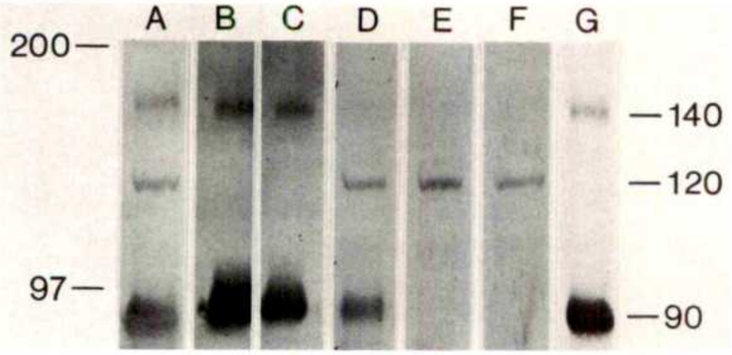

Fig 3. Characterization of platelet antigens recognized by acute-phase serum antibody. Immunoblotting was performed as described in Fig 1. Lane A, control PLA^1-positive platelets; lanes B and C, Lek^a-negative platelets; lane D, platelets from patient's older son (PLA^1-positive); lane E, platelets from patient's younger son (PLA^1-negative); lane F, thrombasthenic platelets; lane G, Bernard-Soulier platelets.

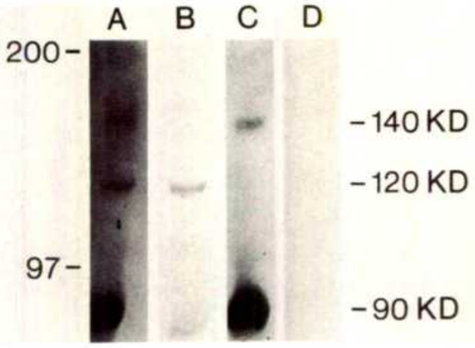

Fig 4. Binding of platelet antibody eluates on immunoblots. Immunoblotting was performed as described in Fig 1, except that platelet eluates at a dilution of 1:50 were substituted for serum. Acute-phase eluate was incubated with control platelets (lane A) or PLA^1-negative platelets (lane B). Convalescent eluate was also incubated with control platelets (lane C) or PLA^1-negative platelets (lane D).

ing of rabbit anti-GP IIb/IIIa antibody to control platelets is shown in lane A. This antibody recognizes two bands corresponding to GP IIb (mol wt 140,000 and 130,000) and a single 90,000-dalton band corresponding to GP IIIa, as previously described.[23] Binding of the patient's acute-phase serum to control platelets preincubated with normal rabbit serum is shown in lane B. Preincubation with control rabbit serum failed to block binding of the patient's antibodies on the blot. In contrast, the blocking experiment using rabbit anti-GP IIb/IIIa followed by the patient's acute-phase serum is shown in lane C. Preincubation with the rabbit antiserum abolished binding of the patient's antibodies to the 140,000-dalton and 90,000-dalton antigens, suggesting that these epitopes are located on GP IIb and GP IIIa. Patient antibody binding to GP 120 was not blocked by the rabbit antiserum. This finding provides further evidence that GP 120 is distinct from GP IIb/IIIa.

Lane D shows the binding of acute-phase serum to reduced platelet proteins. Binding to the GP IIb and PLA^1 epitopes was abolished by reduction of disulfide bonds. Binding to GP 120 in reduced platelets, however, remained unchanged. Thus, GP 120 is distinct from GP IIb/IIIa by both antigenic and conformational studies on immunoblots. Binding of the patient's acute-phase serum to neuraminidase-treated platelets is shown in lane E. Neuraminidase treatment abolished antibody binding to the GP 120 and GP IIb epitopes in these platelets. Antibody binding to PLA^1 was not affected by exposure of platelets to the enzyme, however. These findings suggest that the GP 120 and GP IIb epitopes contain carbohydrate moieties that are important for patient antibody binding to these antigens.

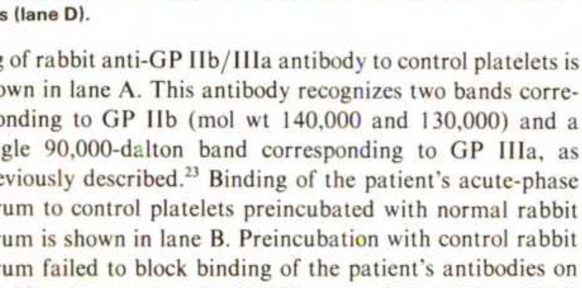

Fig 5. Immunoblots showing rabbit antibody blocking experiment and patient antibody binding to reduced and neuraminidase-treated platelets. Control platelets were incubated with rabbit anti-glycoprotein (GP) IIb/IIIa (lane A), normal rabbit serum followed by acute-phase patient serum (lane B), or rabbit anti-GP IIb/IIIa followed by acute-phase patient serum (lane C). Acute-phase patient serum was also incubated with reduced platelets (lane D), and neuraminidase-treated platelets (lane E). GP IIb is recognized as two bands of mol wt 140,000 and 130,000, whereas GP IIIa has a mol wt of 90,000.

DISCUSSION

The patient described in this article demonstrated several classic features of PTP: (a) Onset of severe thrombocytopenia and bleeding ~1 week after RBC transfusion, (b) history of prior pregnancies, (c) therapeutic response to plasmapheresis, (d) PLA^1-negative platelets and circulating anti-PLA^1 antibody, and (e) HLA B8 positivity.[24] Two theories have been proposed to explain destruction of the patient's own platelets in PTP. Shulman and colleagues[25] and, more recently, Kickler and co-workers[26] postulated an "innocent bystander" reaction whereby anti-PLA^1 antibody binds to soluble PLA^1 antigen that is transfused in stored blood. This immune complex is then adsorbed to PLA^1-negative platelets, causing their destruction. Problems with this hypothesis include the inability to detect circulating PLA^1 antigen–antibody complexes,[25,27] persistence of thrombocytopenia after the disappearance of anti-PLA^1 antibody in at least one case,[3] and the lack of interaction of PLA^1-negative platelets with the postulated immune complex in vitro.[25] In addition, the transfused platelet antigen would have to persist for an extended period, since thrombocytopenia in PTP may last for >2 months.[28,29] A second theory is that PLA^1-positive and PLA^1-negative platelets share other antigenic determinants, such as Lek^a (Bak^a), and that alloantibody production against PLA^1 is accompanied by autoantibody production against these determinants.[1,28,30] Evidence in favor of this hypothesis is lacking at present.[31,32]

Our findings in a single patient with classic PTP have two significant implications. First, they clearly demonstrate that autoantibody as well as alloantibody is present in the acute-phase serum from a patient with PTP, as suggested in a recent study.[28] Second, our results indicate that alloantibodies against more than one platelet antigen may be present in PTP serum and persist after recovery. Thus, these alloantibodies do not appear to be the primary cause of autologous platelet destruction. In contrast, the disappearance of the patient's autoantibody in convalescent serum when the platelet count has become normal suggests that this antibody plays a role in autologous platelet destruction. In preliminary studies of serum from four of five additional patients with PTP, we detected antibody binding to PLA^1-negative control platelets using a flow cytometric technique (R.B. Stricker, L. Corash, M.A. Shuman, unpublished observations). Although this finding is consistent with the presence of non-PLA^1 autoantibodies, it could also be explained by the presence of

HLA antibodies or other alloantibodies that are often detected in PTP.[5,9] We were unable to obtain autologous platelets from these patients to characterize the binding properties of their antibodies. In addition, we did not detect anti-GP 120 antibody by immunoblotting in these additional cases, perhaps due to a delay in sample collection. Thus, it remains to be determined whether transient platelet autoantibody production is a common feature of PTP.

The nature of the previously unrecognized mol wt 120,000 platelet autoantigen is uncertain. Our elution studies indicate that it is a surface antigen and therefore presumably a glycoprotein.[33] In addition, pretreatment of control platelets with neuraminidase abolished antibody binding to the mol wt 120,000 antigen on immunoblots (Fig 5). The GP 120 epitope is present on thrombasthenic platelets, implying that it is distinct from the GP IIb/IIIa complex. The epitope appears to be missing from Bernard-Soulier platelets, suggesting that GP 120 may be related to the GP Ib complex.[34] However, GP 120 is distinct from GP Ib, which has a mol wt of 170,000 daltons (as defined by rabbit anti-GP Ib antibody) in the gel system used in our experiments. GP 120 is also distinct from glycocalicin (mol wt 150,000) and GP V (mol wt 80,000) and may represent a previously undefined component of the GP Ib complex.[34] The GP 120 epitope also appears to be deficient in Lek^a (Bak^a)-negative platelets. Lek^a (Bak^a) is an epitope of GP IIb that is missing in thrombasthenic platelets.[11] In contrast, GP 120 is present in thrombasthenic platelets and is not recognized by a rabbit polyclonal antibody raised against GP IIb, as shown in Fig 5. Thus, the relationship (if any) of GP 120 to Lek^a (Bak^a) is unclear.

Similarly, the GP IIb epitope recognized by the patient's alloantibody is uncharacterized. Evidence that this antigen is a component of GP IIb derives from three observations: First, the antigen's mol wt is identical to the mol wt of an isomer of GP IIb in unreduced gels (Fig 5). Second, binding of the patient's antibody to the 140,000-dalton protein on immunoblots is blocked by prior incubation with rabbit polyclonal antibody against GP IIb, as shown in Fig 5. Third, the epitope is undetectable in platelets from two thrombasthenic patients known to have $<10\%$ GP IIb. The antigen was not detected in platelets from four PLA^1-negative controls and, like PLA^1, it disappeared from control platelets after disulfide bond reduction (Fig 5). The antigen was not detected in platelets from the patient's older son who is PLA^1-positive, or in platelets from two of 20 additional PLA^1-positive patients (data not shown). Thus, the relationship between the GP IIb-associated alloantigen and the PLA^1 alloantigen is uncertain. The GP IIb epitope was also present in Lek^a (Bak^a)-negative platelets, implying that it is distinct from Lek^a (Bak^a). The antigen may, however, be identical to the GP IIb epitope recently defined by serum from another patient with PTP.[35] Additional platelet screening studies are necessary to determine whether this epitope of GP IIb represents a new platelet alloantigen. The epitopes recognized by the anti-GP IIb and anti-GP IIIa antibodies appeared to segregate independently, as evidenced by the fact that both of the patient's sons lacked the GP IIb epitope, although one was PLA^1-positive and the other PLA^1-negative. This observation is consistent with separate genes for GP IIb and GP IIIa.[36]

In summary, we have demonstrated the presence of serum autoantibody directed against a previously undefined platelet antigen in a patient with PTP. We also detected alloantibodies against epitopes of GP IIb and GP IIIa in the same patient. The prevalence of these antibodies in PTP remains to be determined. Effective use of plasmapheresis and intravenous immunoglobulin[28,37,38] should improve patient survival and allow more detailed study of the antibodies involved in the pathogenesis of this enigmatic disease.

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Iron Deficiency and Neutrophil Function: Different Rates of Correction of the Depressions in Oxidative Burst and Myeloperoxidase Activity After Iron Treatment

By Hisashi Murakawa, Catherine E. Bland, Wayne T. Willis, and Peter R. Dallman

The polymorphonuclear granulocyte (PMN) kills ingested bacteria by mechanisms that include myeloperoxidase (MPO) and a sudden increase in oxygen consumption (the oxidative burst), both of which are iron dependent. The magnitude of the oxidative burst and activity of MPO were determined in PMNs during the progression of iron deficiency (ID) and following its treatment in rats. As ID developed, the oxidative burst after zymosan activation was less depressed than the activity of MPO. There was no change in the oxidative burst after activation with phorbol myristate acetate (PMA) or in the generation of superoxide (O_2^-) by NADPH oxidase-containing particles from PMNs. Following iron treatment, impairment of the oxidative burst after zymosan activation was corrected after 1 day. In contrast, the deficit in MPO activity was not corrected until 7 days after initiation of iron treatment. The pattern of recovery in MPO activity after iron treatment corresponded to the prolonged period of maturation of the PMN primary granule since the formation of primary granules, which contain MPO, takes place only in the early, mitotic stages of maturation. The tendency of the PMN to maintain the oxidative burst allows the cell to preserve its capacity for bacterial killing during the progression of iron deficiency.

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IRON DEFICIENCY (ID) results in impairments of immune function, particularly in respect to cell-mediated immunity and the ability of polymorphonuclear granulocytes (PMNs) to kill ingested bacteria.[1-5] The PMN is of particular interest with respect to ID, since the cell has many iron-containing constituents. At least two morphologically and chemically distinct granule populations have been characterized in the cytoplasm of the PMN.[6,7] The primary granule is the only granule that is formed early in maturation; it is produced mainly during the promyelocyte stage and contains myeloperoxidase (MPO).[7] This iron-containing enzyme contributes to the antimicrobial function of the PMN,[8] but the importance of this role is uncertain, since patients with a congenital deficiency of MPO are virtually asymptomatic.[9,10] Other granules, including the specific granule, are formed during and subsequent to the myelocyte stage.[7] The specific granule or a third type of granule is believed to contain cytochrome b, an iron-containing constituent that is believed to be required for the oxidative burst, the marked increase in oxygen consumption that occurs following phagocytosis.[11-14] The oxidative burst plays an important role in bacterial killing, as judged from the marked susceptibility to infection in chronic granulomatous disease, a congenital disorder in which the oxidative burst is absent or decreased.[11-14]

In studies of PMN function in ID children reported by Chandra and Saraya,[15,16] the nitroblue tetrazolium (NBT) test, which reflects the oxidative burst, was decreased. The abnormality was reversed when patients were reinvestigated 4 to 7 days after treatment with iron.[15] Most studies of bactericidal capacity in ID show it to be impaired.[15-20] Reversal of the abnormality was reported within 4 to 7 days by Chandra.[15] Walter and colleagues observed partial recovery after 3 to 5 days and complete recovery by 15 days.[20] Correction of the abnormality was much slower in the study of Yetgin and co-workers,[17] in which bacterial killing was partially improved after 1.5 months of iron treatment and completely reversed after 3 months of therapy. Experimental evidence in rats also showed an abnormal NBT test and decreased MPO activity in ID that had reversed when PMNs were reevaluated 2 months after initiation of iron treatment.[21]

Because the contrasting maturation patterns of the different types of PMN granules might result in correspondingly distinct rates of reversal of abnormalities in PMN enzymes and related functions during recovery from ID, we investigated MPO activity and the oxidative burst, abnormalities associated with the primary granules and the specific granules, respectively, before and after iron administration to rats with ID.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Bantin-Kingman, Fremont, CA) were obtained at weaning (21 days of age) and were randomly assigned to one of two dietary groups, the ID and the control group. ID rats received an ID diet (iron 3 to 9 mg/kg diet); control rats were given the same diet but with added ferric citrate (iron 60 to 63 mg/kg diet). Both diets were otherwise identical in composition to the American Institute of Nutrition diet proposed for growing rats with the exception that cellulose (which has a variable iron content) was omitted.[22] Diets (Teklad, Madison, WI) and distilled water were provided ad libitum.

Protocol. Groups of 5 to 8 rats in each of the diet categories were studied after 1, 2 and 3 weeks of diet; 1, 2, 3, 5, and 7 days after iron administration for the MPO assay; and 1 and 3 days after iron administration for measurement of the oxidative burst. After 3 weeks of the diets (at 6 weeks of age), rats of the ID group received 10 mg of elemental iron as iron dextran (Imferon, Merrell Dow Pharmaceutical, Cincinnati) divided into two intramuscular injections of 0.1 mL each. Control rats received an injection of the same volume of 0.9% saline. Hemoglobin and body weight were measured

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on the day of each experiment. Hemoglobin was determined by the cyanmethemoglobin method (Data Medical, Arlington, TX).

Isolation of rat PMNs. Functionally active PMNs were obtained from the peritoneal cavity 4 hours after intraperitoneal (IP) injection of a casein suspension as described by Lemanske and colleagues.[23] Rats were killed by decapitation, and the peritoneal cavities were lavaged with a total of 40 mL of chilled Dulbecco's phosphate-buffered saline (PBS), pH 7.4, without calcium and magnesium (CMF).[24] The peritoneal exudates were collected separately from each rat and filtered through gauze into a 50-mL sterile centrifuge tube. The cells were kept at 4°C or on ice throughout the isolation procedure.

The PMN concentrates were centrifuged at 400 g for 15 minutes at 4°C. The resulting pellets were treated with 5 mL of ammonium chloride solution for 2 minutes with moderate agitation to lyse RBCs.[25] After 2 minutes, 40 mL of CMF was added to each tube and mixed thoroughly. The cells were then centrifuged and washed three times with CMF.

In a second experiment, PMNs were isolated from the peripheral blood as described by Babior and Cohen[24] with minor modifications. Peripheral blood from male Sprague-Dawley rats was collected in a syringe containing 1 to 1.5 mL of acid-citrate-dextrose solution (ACD). The anticoagulated blood was diluted with the same amount of CMF and mixed with half the amount of 4.5% dextran (mol wt 488,000) in 0.9% saline. The upper layer was removed after the mixture was placed upright for 1 hour at room temperature. The leukocyte-rich plasma was centrifuged, and resulting pellets were treated as described above. The cells were washed twice with CMF, pooled, and suspended in 20 mL of CMF. Then 10 mL of Histopaque-1077 was carefully injected under a 20-mL layer of leukocyte suspension in a 50-mL sterile centrifuge tube using a spinal needle. The tube was centrifuged at 400 g for 20 minutes, and the overlying fluid was aspirated.

The washed neutrophils obtained from either the peritoneal cavity or the peripheral blood were then resuspended in CMF and counted using a Coulter counter model ZBI (Coulter Electronics, Hialeah, FL). Differential counts were performed on Giemsa-stained blood smears to verify that >90% of the cell from the peritoneal cavity and 70% from the peripheral blood in each preparation were PMNs. Cell viability was >95% as determined with trypan blue in each preparation.

MPO was assayed to the method of Migler and DeChatelet with *p*-phenylenediamine as the hydrogen donor.[26] Isolated PMNs in a final concentration of 10^7 cells/mL were subjected to sonication (model W-220, Heat Systemics-Ultrasonic, Plainview, NY) for two 8-second bursts in 0.25% Triton X-100. Disposable polystyrene cuvettes (Fisher Scientific, Pittsburgh) containing 2.75 to 2.92 mL of 10 mmol/L of *N*-[2-acetaamido]-iminodiacetic acid(ADA) pH 6.0, 30 μL of 0.075% H_2O_2, and 25 μL of 1% *p*-phenylenediamine in ethanol (wt/vol) were mixed thoroughly. The reaction was then started by addition of 25 to 200 μL of the PMN suspension (to a total 3.0-mL vol), with a final mixing by rapid inversions of the cuvette. The change in the absorbance at 485 nm was measured using a Hitachi double-beam spectrophotometer (Hitachi, Tokyo) with a chart recorder (Cole-Palmer Instrument, Chicago). The results were expressed as change in optical density per minute per 10^6 cells.

Measurement of oxygen consumption by intact PMNs activated by zymosan or phorbol myristate acetate (PMA) was performed as described by Mackler and colleagues[27] in a polarographic chamber (Rank Bros, Cambridge, England) thermostated to 37°C. Preparation of opsonized zymosan was by the method of Markert and colleagues[28] using serum obtained from male rats of the same strain and from the same supplier as the experimental rats. The opsonized zymosan was washed twice with CMF and then suspended at a concentration of 22.5 mg/mL in CMF. The zymosan was prepared fresh for each day's experiment. The assay mixture contained 0.80 mL of a solution of 0.25 mol/L of sucrose and 0.02 mol/L of Tris(pH 7.4), 0.20 mL freshly thawed rat serum (prepared as described above), 0.60 mL of Hank's solution and 0.20 mL of the intact PMNs suspended in CMF to yield a final concentration of 1.5×10^7 cells/mL in a 2.0-mL total vol. Opsonized zymosan, 0.20 mL (22.5 mg/mL in CMF) or 4 mg of PMA in 0.20 mL of the sucrose was then added to the chamber to activate the cells. The maximal rate of O_2 consumption was determined after the addition. The measurement of O_2^- by NADPH oxidase-containing particles from PMNs was measured by the method of Markert and colleagues.[28]

Materials. Histopaque-1077, casein, zymosan A, *p*-phenylenediamine, Triton X-100, and 30% H_2O_2 were obtained from Sigma, St Louis.

Statistical analysis. Data were analyzed using the unpaired Student's *t* test.

RESULTS

Body weight and hemoglobin concentration. The body weight of the ID rats showed no significant difference after rats were on the diet for 1 week (Table 1). At 2 and 3 weeks, the mean weight of the iron-deficient group was below that of the control group by ~8% and ~20%, respectively. Recent studies under similar conditions have shown that the food intake of iron-deficient rats is essentially equivalent to that of control animals[29,30] and hypothesize that the slower weight gain is due to metabolic alterations in the iron-deficient rat.[30] After iron treatment, the weight of the ID group increased rapidly for 1 to 2 days, but a significant difference of 12% between two groups remained after 7 days.

The ID rats had significantly lower hemoglobin levels as compared with the control rats even after 1 week on the diet. The differences became progressively greater with time, to a mean value that was 38% of the control after 3 weeks on the diet. After iron treatment, the hemoglobin levels of the ID group rose rapidly 1 to 5 days after treatment and were within 15% of control values 7 days after initiation of iron treatment (Table 1).

MPO activity. MPO activity did not differ in the ID group during the first 2 weeks of the regimen as compared with control animals (Table 2). During the progression of ID, the mean MPO activity of the ID group was not significantly reduced until after 3 weeks of diet, when it was significantly depressed, to 53% of the control activity. After iron treatment, the MPO activity of the deficient group did not change

Table 1. Body Weight and Hemoglobin Concentration

Week of Diet or Days After Treatment	Body Weight (g)		Hemoglobin (g/dL)	
	Iron-Deficient	Control	Iron-Deficient	Control
Week 1	90 ± 2	98 ± 2	8.1 ± 0.2*	12.4 ± 0.2
Week 2	132 ± 4*	149 ± 2	6.1 ± 0.3*	13.8 ± 0.1
Week 3 (day 0)	160 ± 3*	201 ± 3	5.3 ± 0.2*	13.8 ± 0.3
Day 1	171 ± 4*	196 ± 3	6.2 ± 0.2*	13.8 ± 0.3
Day 2	185 ± 5	208 ± 7	9.1 ± 0.3*	15.0 ± 0.4
Day 3	186 ± 5*	226 ± 6	9.8 ± 0.2*	13.8 ± 0.3
Day 5	193 ± 5*	237 ± 5	11.6 ± 0.2*	14.6 ± 0.2
Day 7	200 ± 5*	228 ± 8	12.2 ± 0.3*	14.3 ± 0.2

Means ± SEM are shown.

*Significant differences between two groups ($P < .001$).

Table 2. Myeloperoxidase Activity in PMNs During Progression of Iron Deficiency and Following Iron Treatment

Weeks of Diet or Days of Iron Treatment	Myeloperoxidase, Δ OD/min/10^6 PMNs			
	Rats per Group	Iron-Deficient Rats	Control Rats	Percent of Control
1 Week	8, 7	0.172 ± 0.014	0.165 ± 0.015	104
	8, 8	0.160 ± 0.020	0.180 ± 0.016	89
2 Weeks	8, 7	0.147 ± 0.011	0.177 ± 0.024	83
3 Weeks	8, 7	0.209 ± 0.023*	0.396 ± 0.057	53
1 Day	8, 7	0.172 ± 0.013†	0.298 ± 0.021	58
2 Days	8, 5	0.259 ± 0.018*	0.435 ± 0.032	60
3 Days	8, 8	0.141 ± 0.009†	0.265 ± 0.022	53
5 Days	7, 7	0.216 ± 0.013*	0.270 ± 0.021	80
	8, 8	0.232 ± 0.013†	0.350 ± 0.031	66
7 Days	8, 8	0.275 ± 0.018	0.242 ± 0.016	114

PMNs, polymorphonuclear granulocytes.

Additional groups of deficient and control rats were studied at two of the time points. Iron was administered to iron-deficient rats after 3 weeks of iron-deficient diet.

*$P < .01$.

†$P < .001$.

for 3 days and did not attain control levels until 7 days after iron treatment.

The oxidative burst. The changes in the rates of oxygen consumption of PMNs after stimulation with zymosan and PMA are shown in Table 3. No significant differences were found after 1 and 2 weeks of diet. After 3 weeks, however, there was a small but significant depression in oxygen consumption after zymosan activation in the ID group that was confirmed in an additional experiment. One and 3 days after iron treatment, the rate of oxygen consumption of the deficient PMNs was equal to that of the control PMNs. There was no difference in oxygen consumption after activation with PMA between iron-deficient and control rats studied after 3 weeks of the dietary regimens. O_2^- production by NADPH oxidase-containing particles from PMNs was not depressed after 3 weeks of the iron-deficient regimen (Table 4).

Peripheral blood PMNs. PMNs obtained from the peripheral blood were used to validate the results obtained on peritoneal PMNs in a second and smaller experiment on two randomly assigned groups of rats that had been given the ID diet for 3 weeks. One group had been given intramuscular (IM) Imferon injection on the previous day, and the other group was untreated. The activity of MPO was not changed 1 day after treatment, whereas the rate of oxygen consumption after zymosan activation was significantly increased (Fig 1). Absolute values for oxygen consumption were considerably lower than in PMNs from peritoneal exudate, possibly due to the different method of isolation.

Table 3. Oxygen Consumption After Activation of PMNs With Zymosan or PMA

Weeks of Diet or Days of Iron Treatment	Rats per Group	Oxygen Consumption, nmol O_2/min/10^6 PMNs		
		Iron-Deficient Rats	Control Rats	Percent of Control
Zymosan				
1 Week	8, 8	1.75 ± 0.15	1.73 ± 0.16	101
2 Weeks	8, 7	4.61 ± 0.22	4.43 ± 0.34	104
3 Weeks	9, 9	3.80 ± 0.28*	4.73 ± 0.32	80
	6, 7	4.32 ± 0.34*	5.29 ± 0.18	82
1 Day	8, 7	5.21 ± 0.35	4.71 ± 0.40	110
3 Days	8, 8	5.41 ± 0.39	5.16 ± 0.32	105
PMA				
3 Weeks	8, 7	4.51 ± 0.21	4.40 ± 0.23	103
1 Day	7	4.41 ± 0.15	—	100

PMNs, polymorphonuclear granulocytes; PMA, phorbol myristate acetate.

*$P < .05$.

DISCUSSION

Clinical studies suggest that bacterial killing by PMNs depends primarily on the oxidative burst rather than on MPO. This is evident from a comparison between chronic granulomatous disease, an inherited abnormality in the O_2^--forming oxidase or its activating system, and hereditary MPO deficiency. Chronic granulomatous disease, usually fatal, is characterized by recurrent infections caused by an inability to generate an oxidative burst to kill invading microorganisms.[31] In contrast, the antibacterial defense in patients with MPO deficiency is relatively well preserved, with little evidence of morbidity other than a susceptibility to infection with Candida.[9,10]

Our results in the ID rat indicate that the oxidative burst is less impaired than MPO activity after 3 weeks of an iron-deficient regimen. This presumably preserves the most vital function of the PMN. Mackler and co-workers[27] also reported a more profound depression of MPO than of the

Table 4. Superoxide (O_2^-) Production by NADPH Oxidase-Containing Particles from PMNs

Group	Rats per Group	nmol O_2^- Produced/min/10^6 PMNs	Percent of Control
Control, 3 weeks	7	3.9 ± 0.3	100
Iron-deficient, 3 weeks	8	4.4 ± 0.4	113
Iron-deficient, 1 day treated	8	4.4 ± 0.3	113

PMNs, polymorphonuclear granulocytes.

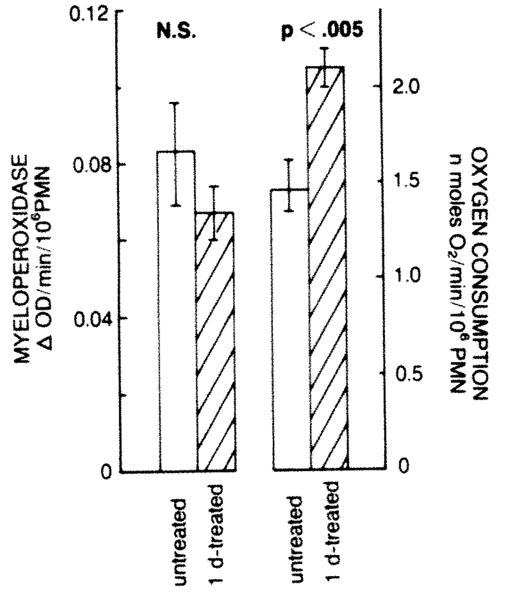

Fig 1. Myeloperoxidase activity and oxygen consumption after zymosan stimulation in 6 to 7 polymorphonuclear granulocyte (PMN) concentrates per group obtained from peripheral blood of 3 rats each in rats given an iron-deficient diet for 3 weeks, either saline treated (open bars) or iron treated (hatched bar). SEM is shown.

oxidative burst of PMNs in iron-deficient rats. They did, however, find a significant depression with PMA activation after 6 weeks of the dietary regimens. Surprisingly, PMN cytochrome b, which is thought to be essential for the oxidative burst,[30-32] was normal in concentration.[27]

The generation of an oxidative burst is normally initiated by phagocytosis, which is followed by activation of the pentose shunt with a concomitant production of NADPH. The NADPH is then oxidized to generate O_2^-, which initiates a sequence of steps that result in bacterial killing. Zymosan is a particulate activator that mimics the effect of bacteria and other invasive organisms. It consists of yeast cell walls and must be ingested by the PMN to generate an oxidative burst. In contrast, PMA is a soluble stimulus that does not require phagocytosis. Our finding of a decreased oxidative burst in response to zymosan, but not following PMA, suggests that phagocytosis may have been a limiting factor. Also in accord with this hypothesis is the maintenance of normal O_2^- production by PMN NADPH oxidase (Table 4). Additional evidence that impaired phagocytosis might explain a decrease in the zymosan-activated oxidative burst is the observation by Moore and Humbert[21] that PMNs from iron-deficient rats show a slightly diminished phagocytosis of yeast cells (*C albicans*). The return to normal of the zymosan-activated oxidative burst within 1 day of iron administration might then indicate that such a mild defect in phagocytosis is rapidly reversed.

Our finding that the mild impairment of the oxidative burst after zymosan activation was corrected 1 day after iron treatment is compatible with Chandra's finding that NBT test in patients was normal 4 to 7 days after iron was given IM.[15] In the study of Walter and colleagues, the abnormality in bacterial killing was partially improved after 3 to 5 days and was completely corrected 15 days after oral iron treatment.[18] These results suggest somewhat slower recovery of the oxidative burst that might be due to the species difference, duration of prior iron deficiency, methodology, or the route of iron administration.

The pattern of PMN recovery in respect to MPO activity after iron treatment corresponds to the maturation of the primary granule. Reversal of deficient MPO production apparently takes place only in the early stages of PMN maturation, since recovery after iron treatment is not apparent until 5 days and not complete for 7 days. Kinetic studies of PMN maturation in the rat after in vivo labeling of the proliferating cells in the tibial bone marrow with (^3H)thymidine show that 3 days elapse before peak labeling occurs in released PMNs.[35] MPO is synthesized during the early, mitotic stages of PMN maturation. The delay in reversal of MPO deficiency after iron treatment indicates that adequate iron must be present when the primary MPO-containing granules are produced and that correction of the deficit in MPO cannot take place at a later stage of cell maturation.

Our results indicate that the PMN in the ID rat tends to preserve its most vital function for bacterial killing and to correct this abnormality rapidly with iron treatment. Whether the sequence of recovery is the same in ID patients remains to be determined by more detailed investigations during the first 2 weeks of therapy.

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Cytosolic Free Calcium Levels in Sickle Red Blood Cells

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In this study, we used a recently developed nuclear magnetic resonance (NMR) technique to measure ionized calcium in sickle erythrocytes. The NMR technique, which involves ^{19}F NMR studies of a fluorinated calcium chelator quinMF, [2-(2-amino-4-methyl-5-fluorophenoxy)methyl-8-aminoquinoline-*N,N,N',N'*-tetraacetic acid] provides a novel approach to the study of ionized calcium in erythrocytes since the presence of hemoglobin precludes the use of fluorescent calcium indicators. The mean value for ionized calcium in oxygenated sickle erythrocytes was 18 ± 2 nmol/L (SE). Experiments with normal RBCs gave a mean value of 21 ± 2 nmol/L (SE). After 1 hour of deoxygenation, mean values for ionized calcium in sickle erythrocytes did not increase as compared with values obtained under oxygen. To investigate whether deoxygenation stimulated endocytosis, sickle erythrocytes were deoxygenated for 1 hour in the presence of impermeant FBAPTA (1,2 *bis*-(2-amino-5-fluorophenoxy) ethane *N,N,N',N'*-tetraacetic acid). Cells were then separated from the extracellular medium and assayed for the presence of FBAPTA; they had incorporated significant quantities of the extracellular FBAPTA. This incorporation was not observed with normal erythrocytes. These data are consistent with at least a portion of the elevation in total cell calcium in sickle erythrocytes arising as a consequence of an endocytotic process in which extracellular calcium ions are incorporated into vesicles. Additional experiments show that these intracellular vesicles accumulate Ca^{2+} on further deoxygenation, consistent with a transient increase in ionized cell calcium. These studies represent the first use of NMR spectroscopy to evaluate endocytotic processes.

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THERE IS A five- to tenfold elevation of total calcium in sickle erythrocytes as compared with normal RBCs, and isotopic calcium influx is increased in deoxygenated sickle erythrocytes, an effect that does not occur when normal RBCs are deoxygenated.[1,2] Elevation of erythrocyte total calcium, if it is not sequestered, should contribute to the pathogenesis of sickling by activating K^+ specific channels in the cell membrane.[3,4] Activation of these channels would cause loss of K^+ and water, which in turn would enhance sickling because cell dehydration promotes the gelation of hemoglobin S.[4] Lew and colleagues[5] recently demonstrated by electron-microprobe analysis that sickle erythrocytes have intracellular vesicles containing mmolar levels of calcium.

Although these studies show that Ca^{2+} is sequestered in sickle erythrocytes, they do not determine whether an increase in cytosolic free calcium (Ca_i^{2+}) occurs in sickle erythrocytes either under basal conditions or during deoxygenation. Indeed, recent studies of Bookchin and co-workers[6] reported indirect evidence for a transient increase in calcium-induced K-channel in these cells. An indirect, nonkinetic method used to calculate Ca_i levels in erythrocytes suggests that under basal conditions Ca_i^{2+} levels in sickle and in normal erythrocytes do not differ significantly.[7] Ca_i^{2+} levels were not, however, measured during deoxygenation, a condition that stimulates ^{45}Ca uptake in sickle erythrocytes.[2] Furthermore, although Lew and colleagues[5] demonstrated the presence of calcium-containing vesicles in sickle erythrocytes, little is known about the formation of these vesicles and about the origin of the calcium contained within them.

In the present study, using a nuclear magnetic resonance (NMR) technique that allows direct measurement of Ca_i^{2+}, we determined a value of 18 ± 2 nmol/L (SE) for ionized calcium in well-oxygenated sickle RBCs. This value does not differ significantly from values obtained with normal human erythrocytes. No increase in ionized calcium is found in sickle erythrocytes on deoxygenation. Moreover, deoxygenation of sickle erythrocytes leads to incorporation of an extracellular impermeant chelator of calcium, suggesting that deoxygenation stimulates endocytosis. In addition, by deoxygenating sickle erythrocytes that had previously incorporated FBAPTA, we observed an increase in the calcium level in these vesicles, demonstrating dynamic Ca^{2+} uptake by the vesicles on deoxygenation.

MATERIALS AND METHODS

Cell preparation. Samples were obtained from normal controls or patients with homozygous sickle cell anemia who had not been transfused for a minimum of 6 months. The samples were drawn into heparinized-vacuum tubes and subsequently diluted in a modified saline solution containing (in mmolar): NaCl 132, $MgSO_4 \cdot 7H_2O$ 0.8, KCl 5.4, $CaCl_2$ 1.25, Hepes 5.0, adjusted to pH 7.4 with Tris base. The cells were then centrifuged at 1,000 g for 7 minutes, and the supernatant and buffy coat were removed. For measurement of Ca_i^{2+}, cells were loaded with the fluorinated calcium chelator, quinMF, by incubating them at a hematocrit of 3% to 4% in the modified saline solution, with 50 μmol/L of the acetoxymethyl ester of quinMF (quinMF-AM) for 20 minutes at 37°C. The cells were then centrifuged at 1,000 g for 7 minutes and, after resuspension in the modified saline, were incubated for an additional 60 minutes to allow the cells to recover from the loading procedure. For the NMR studies, the cells were resuspended at a 40% hematocrit in modified saline.

A byproduct of loading erythrocytes with quinMF-AM is formaldehyde, which can inhibit the glycolytic enzyme glyceraldehyde

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3-phosphate dehydrogenase as a consequence of an increased NADH/NAD ratio.[8] This can lead in turn to ATP depletion. To ameliorate the effect of formaldehyde, Na pyruvate, 5 mmol/L, was added to the modified saline solution. Na pyruvate generates NAD through the enzyme lactate dehydrogenase so that the cells maintain normal ATP levels despite the presence of formaldehyde.[8] We previously showed that, in the presence of pyruvate, loading RBCs with a similar calcium chelator (FBAPTA) caused only a slight (<20%) fall in ATP.[9]

Measurement of Ca_i. Cytosolic free Ca^{2+} (Ca_i^{2+}) was measured by ^{19}F NMR spectroscopy[10] using a newly described fluorinated calcium chelator, quinMF.[11] QuinMF has a much lower dissociation constant than any of the fluorinated chelators originally developed by Smith and co-workers,[10] and it is therefore more useful for measuring Ca_i in erythrocytes.[11] The erythrocytes were suspended in a 20-mm NMR tube fitted with an air-driven stirrer to keep the cells in suspension and to facilitate oxygenation and deoxygenation.[9] The cells were gassed with either 100% O_2 or 100% N_2, resulting in no pH change with the Hepes-Tris buffer. Once inside the cell, the quinMF is in slow chemical exchange with Ca^{2+}. This gives two peaks in the NMR spectrum, one arising from uncomplexed quinMF and another from Ca^{2+} complexed quinMF (Ca-quinMF). Ca_i^{2+} can be calculated using the following equation: $Ca_i^{2+} = K_D[\text{Ca-quinMF}]/[\text{quinMF}]$.

Under conditions of nonsaturation or of equal saturation of both ^{19}F resonances, the concentrations of Ca-quinMF and quinMF are proportional to the area under their respective peaks. Peak area was determined by cutting and weighing. The previously determined K_D of 63 nmol/L was used.[11] The level of loading with quinMF was assessed by comparing the area of the quinMF and Ca-quinMF peaks to the area of a known concentration of the standard 6-fluorotryptophan. These studies were performed on a Nicolet (Fremont, CA) NT-360 spectrometer using a 20-mm broad-band Nicolet probe. Observation was made through the decoupler coil, which was tuned to 339.7 MHz for the ^{19}F studies. The sample was shimmed on H_2O, and we routinely obtained a (nonspinning) line width at half height of ~0.1 ppm. A 40° pulse angle, a 500-μs delay and a 127-ms acquisition time were used for these ^{19}F studies. EGTA was added to correct for any extracellular calcium indicator (either FBAPTA or quinMF). Addition of excess EGTA will bind extracellular Ca^{2+}; therefore, any extracellular chelator that is complexed will shift to the uncomplexed form.[9]

Estimation of cell lysis. The percentage of cell lysis was calculated by measuring the hemoglobin (Hb) concentration in the supernatant and comparing this with the total cell Hb concentration. Hb concentration was measured spectrophotometrically at 540 nm.[12]

Materials. The methyl ester of quinMF was synthesized as described,[11] and Molecular Probes (Junction City, OR) effected the conversion to the aceytoxymethyl ester of quinMF. The potassium salt of FBAPTA was purchased from Molecular Probes. All other chemicals were obtained from Sigma.

RESULTS

We were initially interested in determining whether Ca_i^{2+} levels increased in sickle erythrocytes particularly during deoxygenation. FBAPTA has been used previously[9,11,15] to measure Ca_i^{2+} in erythrocytes. The accuracy of the Ca_i^{2+} determination in erythrocytes is limited, however, due to the mismatch between the basal level of Ca_i^{2+} and the binding constant of FBAPTA for Ca^{2+}[11]; thus, even for a 1 mmol/L loading of chelator, the signal of Ca-FBAPTA corresponds to <0.1 mmol/L. This leads to the further complication that at such low fractional levels of complexation, a small amount of cell lysis can lead to a large error in the calculated value of Ca_i^{2+} due to erroneous inclusion of a contribution from the extracellular chelator complexed with extracellular Ca^{2+} present at much higher levels. For these reasons, in this study we used a new fluorinated calcium chelator, quinMF, which has a lower dissociation constant for calcium (K_D = 63 nmol/L).

Figure 1, spectrum A (lower trace), shows a representative fluorine NMR spectrum of quinMF-loaded, oxygenated sickle erythrocytes. Based on data from eight similar experiments (corrected for extracellular quinMF) from seven different donors, cytosolic free Ca^{2+} (Ca_i^{2+}) values averaged 18 ± 2 nmol/L, a level not significantly different from a control value of 21 ± 2 nmol/L obtained using quinMF to measure Ca_i^{2+} in normal erythrocytes. Spectrum B shows the ^{19}F spectrum following 30 minutes of deoxygenation. Figure 1B shows an increase in the linewidth of the resonances, which is expected based on the paramagnetism of deoxygenated hemoglobin.[14] The increase in the ratio of Ca-quinMF to quinMF can reflect either an actual increase in the Ca_i^{2+} level or the presence of extracellular calcium complexed quinMF secondary to cell lysis. The latter interpretation is suggested by the fact that the resonance corre-

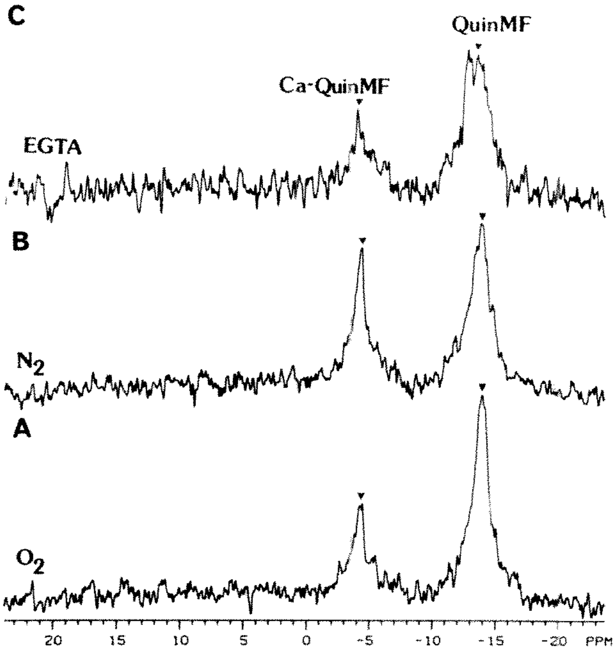

Fig 1. ^{19}F spectra of fluorinated calcium chelator 2-(2-amino-4-methyl-5-fluorophenoxy)methyl-8-aminoquinoline-N,N,N',N'-tetraacetic acid (quinMF)-loaded sickle erythrocytes. (A) Spectrum shows oxygenated erythrocytes (5,000 acquisitions, 10-minute accumulation). Values for control Ca_i^{2+} level were obtained from similar spectra that had been corrected for extracellular quinMF. The suspension was then deoxygenated with 100% N_2 for 30 minutes and spectrum shown in (B) was accumulated for 10 minutes under 100% N_2 (5,000 acquisitions). (C) Spectrum shows a 10-minute accumulation of the same deoxygenated cells to which 10 mmol/L of EGTA was added.

sponding to Ca-quinMF in Fig 1B appears as a relatively sharp resonance superimposed on a broader resonance, potentially reflecting contributions from extracellular and intracellular Ca-quinMF, respectively. To distinguish further between these two possibilities, EGTA was added to the cell suspension and, as shown in spectrum 2C, the addition of EGTA slightly reduced the Ca-quinMF peak. Thus, the increase in the Ca-quinMF peak was due to an increase in extracellular quinMF. In eight experiments with EGTA used to correct for extracellular FBAPTA, Ca_i^{2+} averaged 21 ± 2 nmol/L after deoxygenation. Similar experiments performed on FBAPTA-loaded sickle erythrocytes gave qualitatively similar results, consistent with the suggestion that deoxygenation does not significantly increase Ca_i^{2+}. The levels of Ca_i^{2+} under both basal conditions and deoxygenation are higher in FBAPTA-loaded cells than in quinMF-loaded cells, however. Reasons for this difference are addressed in the Discussion section.

During deoxygenation, a sample of cells was taken and fixed with a 0.2% solution of glutaraldehyde. Microscopic examination of 500 erythrocytes showed that >80% were in the sickled conformation. Using ^{31}P NMR, we also confirmed that deoxygenation of sickled cells caused no decrease in cell ATP (data not shown). Identical deoxygenation experiments were performed with quinMF-loaded normal erythrocytes. After deoxygenation, Ca_i^{2+} of normal erythrocytes averaged 17 ± 4 nmol/L (n = 3), a value not significantly different from either oxygenated normal or deoxygenated quinMF-loaded sickle erythrocytes.

We were also interested in investigating whether deoxygenation stimulated formation of the intracellular vesicles observed by Lew and colleagues[5] using electron-probe microanalysis. To test this hypothesis, cells (that had not been loaded with the acetoxymethyl ester of FBAPTA) were incubated with the ionized form of FBAPTA, which cannot permeate the membrane. If deoxygenation stimulates endocytosis, FBAPTA, although impermeant, should be detected in the sickle erythrocytes. Results are shown in Fig 2. The initial spectrum, (Fig 2A), was taken with 10 mmol/L of FBAPTA and 6.0 mmol/L of $CaCl_2$ in the incubating medium, so that the ratio of CaFBAPTA/FBAPTA was 1.5/1.0. The high ratio of signal to noise reflects the high concentration of FBAPTA and the large extracellular volume. The cells were then deoxygenated for 1 hour at 37°C, washed twice in the modified saline solution at room air, and resuspended in the modified saline solution containing 1.25 mmol/L of $CaCl_2$ (room air). The ^{19}F NMR spectrum (Fig 2B) was then obtained. In this spectrum, both CaFBAPTA and FBAPTA peaks were present, although the ratio of the CaFBAPTA resonance to FBAPTA was larger than in the spectrum shown in Fig 2A. Because excess Ca was present extracellularly, the free FBAPTA peak must have been intracellular. To demonstrate further the presence of endocytosis as opposed to residual extracellular chelator, excess EGTA (20 mmol/L) was added to the incubation medium (spectrum shown in Fig 2C). EGTA shifted some but not all of the resonance intensity from the Ca-FBAPTA position to the free FBAPTA position, confirming that a fraction of the FBAPTA was intracellular in the form of Ca-FBAPTA. The ratio of the resonance intensity of the FBAPTA peak in the spectrum shown in Fig 2B to that in spectrum A, indicates that 0.4% of the CaFBAPTA was sequestered intracellularly. Similarly, the ratio of the CaFBAPTA peak of the spectrum shown in Fig 2C to that of spectrum A was 0.6%. Similar results were obtained after three repetitions of this study.

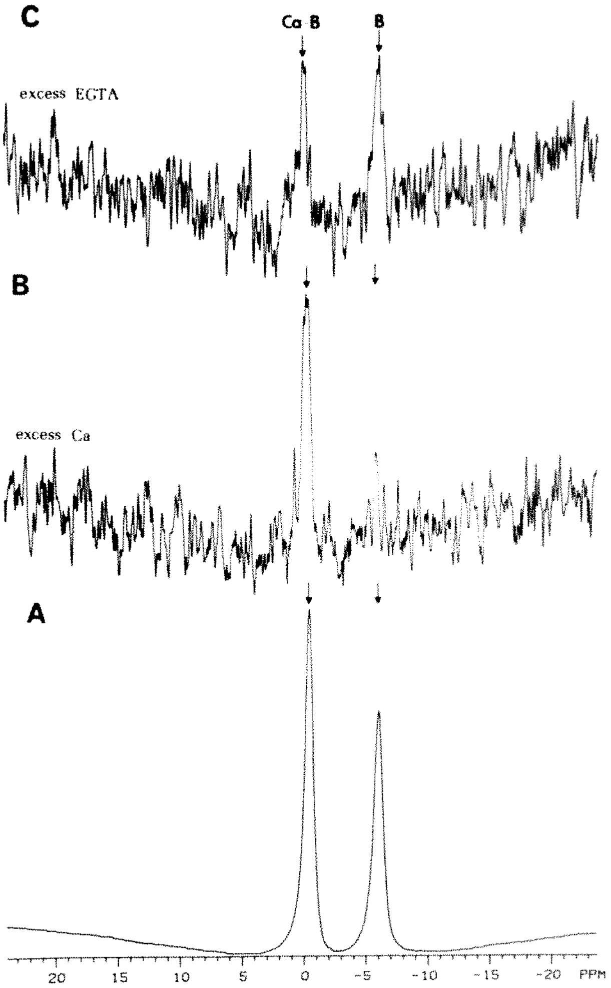

Fig 2. Endocytosis of 1,2 *bis*-(2-amino-5-fluorophenoxy)ethane*N,N,N',N'*-tetraacetic acid (FBAPTA) by sickle erythrocytes. Spectrum (A) shows sickle erythrocytes incubated with impermeant FBAPTA (10 mmol/L) plus sufficient extracellular calcium to give a ratio of CaFBAPTA/FBAPTA of 6/4. The suspension was deoxygenated for 1 hour and then washed twice in modified saline. The cells were resuspended in modified saline containing 1.25 mmol/L of $CaCl_2$ and the spectrum (B) was accumulated. Excess EGTA (20 mmol/L) was then added and another spectrum (C) was accumulated. All spectra were 5,000 acquisitions. CaB, CaFBAPTA; B, FBAPTA.

In Fig 3, using normal erythrocytes, spectra were obtained under conditions similar to those described for Fig 2. The cells were initially suspended in media containing 10 mmol/L of FBAPTA with 8 mmol/L of $CaCl_2$ to give a ratio of CaFBAPTA/FBAPTA of 4/1 (Fig 3A). Subsequent to a 1-hour period of deoxygenation and two washes, the cells

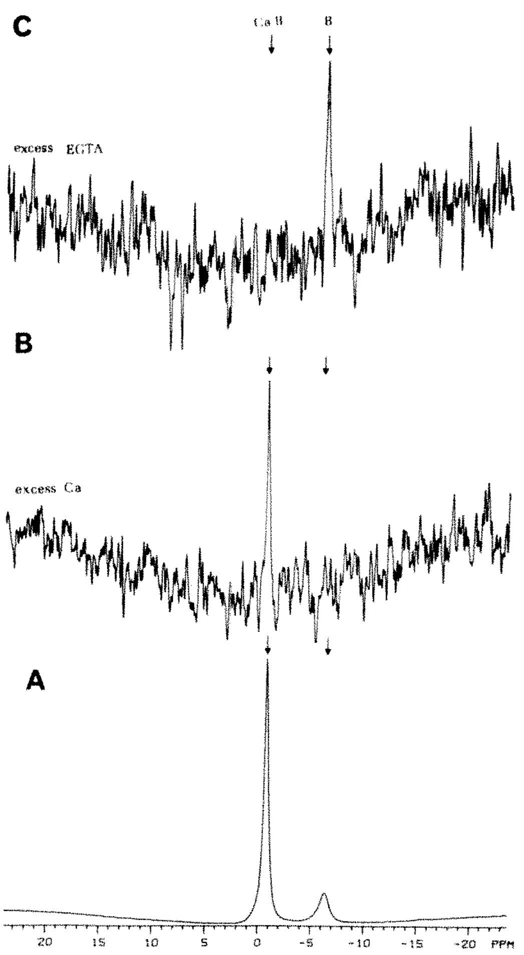

Fig 3. Endocytosis of 1,2-*bis*(2-amino-5-fluorophenoxy)ethane *N,N,N',N'*-tetraacetic acid (FBAPTA) by normal erythrocytes. (A) Spectrum shows normal erythrocytes incubated with impermeant FBAPTA (10 mmol/L) plus sufficient extracellular calcium to give a ratio of CaFBAPTA/FBAPTA of 8/2. The RBCs were deoxygenated for 1 hour and then washed twice in modified saline. Spectra (B) and (C) were then accumulated with excess calcium and excess EGTA, respectively. All spectra were 5,000 acquisitions. Ca-B, CaFBAPTA; B, FBAPTA.

were resuspended in modified saline containing 1.25 mmol/L $CaCl_2$ (Fig 3B). No resonance corresponding to free FBAPTA was observed. The addition of excess EGTA resulted in elimination of the CaFBAPTA resonance and the simultaneous appearance of the free FBAPTA resonance, consistent with the interpretation that all the FBAPTA was extracellular. Similarly, if oxygenated sickle erythrocytes are incubated with FBAPTA, incorportion of FBAPTA does not occur.

The data presented suggest that on deoxygenation, endocytotic vesicles are formed that incorporate extracellular medium, thereby increasing total cell calcium. A final experiment was designed to test the possibility that deoxygenation causes small transient increases in intracellular calcium, which is actively pumped into and sequestered by endocytotic vesicles. For this study, sickle erythrocytes were deoxygenated in a medium in which the ratio of CaFBAPTA/FBAPTA was adjusted to 1/15 (15 mmol/L of FBAPTA with 1 mmol/L of $CaCl_2$). After incorporation of CaFBAPTA/FBAPTA (1:15) into the vesicles, the cells were washed and resuspended in calcium-containing saline. The erythrocytes were then deoxygenated for a second time while ^{19}F spectra were observed. The spectrum in Fig 4A shows the ratio of extracellular CaFBAPTA/FBAPTA present when the cells were sickled by deoxygenation for 1 hour. The cells were then washed twice and resuspended in saline containing 1.25 mmol/L of $CaCl_2$. The spectrum shown in Fig 4B, obtained under oxygen, revealed a peak correspond-

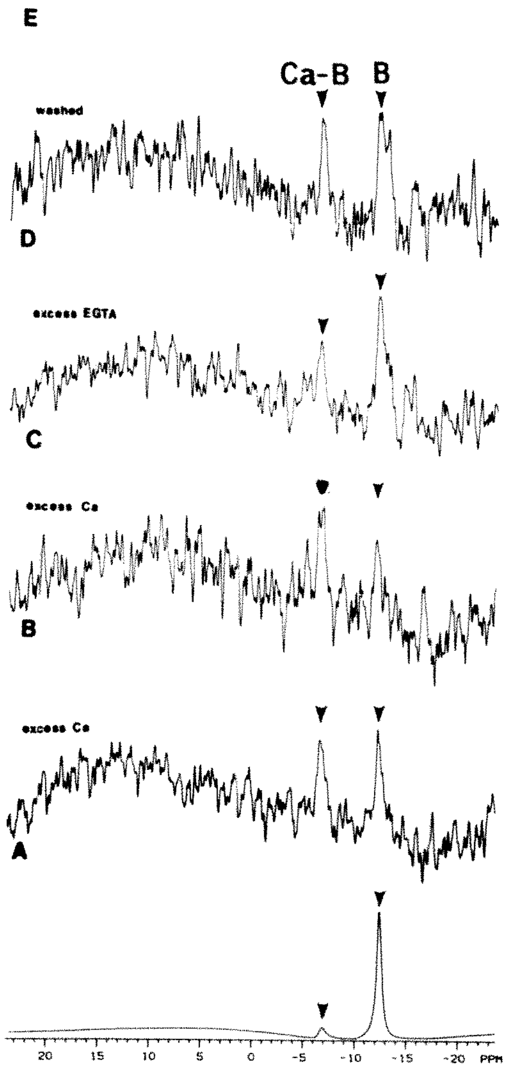

Fig 4. Calcium uptake into endocytotic vesicles during deoxygenation. (A) Spectrum shows erythrocytes incubated with 15 mmol/L of impermeant 1,2 *bis*-(2-amino-5-fluorophenoxy)ethane *N,N,N',N'*-tetraacetic acid (FBAPTA) plus calcium to give a ratio of CaFBAPTA/FBAPTA of 1/15. Cells were deoxygenated for 1 hour, washed and resuspended in an oxygenated buffer containing excess extracellular calcium, and then spectrum B was accumulated. (C) Spectrum was accumulated after the cells were deoxygenated for a second time (20 minutes) in the presence of Ca^{2+}. Following addition of excess EGTA to the deoxygenated suspension, spectrum shown in (D) was accumulated. Cells were then washed and resuspended in excess Ca^{2+}, and spectrum shown in (E) was obtained.

ing to uncomplexed FBAPTA despite the addition of 1.25 mmol/L of $CaCl_2$ to the suspending medium. This finding indicates that FBAPTA was incorporated into the endocytotic vesicle. The spectrum shown in Fig 4C, taken during a second deoxygenation period, shows that resickling caused an increase in the ratio of CaFBAPTA/FBAPTA to 1/1.5. In this second deoxygenation, we deoxygenated until we saw an increase in the CaFBAPTA peak; this time period (20 to 40 minutes) varied from donor to donor. To confirm that this increase was due to Ca uptake into the vesicles and not to cell lysis and subsequent leakage of FBAPTA into the extracellular high Ca medium, we added excess EGTA. In the spectrum shown in Fig 4D, even with the FBAPTA concentration overestimated due to a pool of extracellular FBAPTA (in excess EGTA, extracellular FBAPTA would be uncomplexed), the ratio of CaFBAPTA/FBAPTA increased. This was confirmed in the spectrum in Fig 4E, which shows the intravesicular ratio of FBAPTA/CaFBAPTA following washing and resuspension of the cells in modified saline.

DISCUSSION

NMR studies using fluorinated calcium chelators provide a novel approach to the direct measurement of ionized calcium in human erythrocytes.[9,15] The technique, originally described by Smith and co-workers[10] and used to measure Ca_i^{2+} in thymocytes, is based on the detection of two ^{19}F resonances corresponding to free and calcium-complexed intracellular chelator. Ionized Ca values, calculated using the area under the ^{19}F resonance peaks and the K_D of FBAPTA, range from 30 to 70 nmol/L for normal erythrocytes.[9,15] The principal limitation encountered arises from a small amount of cell lysis (1% to 3%), which results in FBAPTA in the extracellular medium. With $CaCl_2$ present in the incubating media, any extracellular FBAPTA will be entirely in the CaFBAPTA form, leading to an overestimation of the Ca-FBAPTA peak and thereby to an overestimation of the calculated level of Ca_i^{2+}. Because FBAPTA has a K_D more than tenfold above basal Ca_i^{2+} levels, the Ca-FBAPTA peak is less than one-tenth of the FBAPTA peak; therefore, small amounts of fluorinated chelator leakage produce a large percentage of increase in the Ca-FBAPTA peak. Because of these difficulties, we developed new fluorinated calcium chelators with tighter calcium binding.[11] QuinMF has a K_D of 63 nmol/L and, when loaded into normal erythrocytes, the calculated level of Ca_i^{2+} ranges from 15 to 30 nmol/L.[11] The Ca_i^{2+} values calculated using FBAPTA are probably overestimated due to slight errors in the estimation of extracellular FBAPTA.

Our experiments have used this NMR technique to investigate calcium homeostasis in sickle erythrocytes. Total calcium measurements in sickle erythrocytes are substantially elevated as compared with those of normal erythrocytes. The experiments of Lew and colleagues[5] elegantly demonstrate that a considerable amount of Ca is sequestered in sickle erythrocytes. These data, however, do not provide information on whether an increase in Ca_i^{2+} occurs during deoxygenation of sickle erythrocytes. Our studies show that Ca_i^{2+} in well-oxygenated sickle erythrocytes is not significantly different from values of Ca_i^{2+} measured in normal erythrocytes. Thirty minutes of deoxygenation give a slight but not significant increase of Ca_i^{2+} in sickle erythrocytes, although small transient elevations of Ca_i^{2+} during deoxygenation could be missed, since we are signal-averaging for 10 minutes.

It is of interest to study the stimulus for the formation of these vesicles[16] as well as to investigate the means by which these vesicles sequester Ca^{2+}. Data presented in this article showing cellular incorporation of impermeant FBAPTA on deoxygenation suggest that these calcium-containing vesicles are formed by endocytosis of the cell membrane and that during endocytosis they envelop extracellular Ca^{2+}. Similar incorporation could not be demonstrated with either oxygenated sickle erythrocytes or deoxygenated RBCs from normal control subjects, suggesting that only deoxygenation of sickle erythrocytes stimulates endocytosis. Incorporation of FBAPTA in sickle cells cannot be due to trapping of FBAPTA between the cells, since: (a) endocytosis could not be demonstrated in normal cells, and it has been shown that the trapped extracellular space is the same in comparisons of oxygenated normal and sickle cells or deoxygenated normal and sickle cells[17]; and (b) the cells were washed twice, and a portion of FBAPTA was not shifted by addition of extracellular Ca^{2+} or EGTA.

Data presented also support the idea that intracellular vesicles can actively accumulate calcium. Such accumulation was demonstrated by deoxygenating sickle erythrocytes that had previously incorporated FBAPTA. When we resickle these cells that have incorporated FBAPTA, we observe a sufficient increase in the ratio of CaFBAPTA/FBAPTA to indicate a 600% increase in calcium in these vesicles. These data suggest that deoxygenation stimulates Ca^{2+} uptake into these intracellular vesicles, consistent with a transient increase in Ca_i.

These studies indicate that despite the high total calcium that has consistently been observed in sickle RBCs the ionized calcium in the cytosol is not different from that of normal cells. Deoxygenation of sickle RBCs does increase calcium permeability and may result in a slight transient increase in Ca_i, but this excess calcium is rapidly pumped into and sequestered by intracellular, endocytotic vesicles and is pumped out of the cytoplasm by CaATPase. In this manner, such vesicles may have an important role in protecting the sickle erythrocyte from the adverse effects known to result from an increase in intracellular calcium. These studies leave open the possibility, however, that despite the capacity of the vesicles for calcium accumulation, brief transients may exist in intracellular calcium and activate a K^+ channel, leading to ion and water loss from the sickle erythrocyte.

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FVIII Inhibitor IgG Subclass and FVIII Polypeptide Specificity Determined by Immunoblotting

By Carol A. Fulcher, Sytske de Graaf Mahoney, and Theodore S. Zimmerman

We used immunoblotting of purified factor VIII coagulant protein (FVIII) to localize FVIII inhibitor epitopes in 76 inhibitor plasmas to either the 92-kd FVIII polypeptide (and its 54-kd and/or 44-kd thrombin fragments), the 80-kd polypeptide (and its 72-kd thrombin fragment), or both of these polypeptides. We also used immunoblotting to examine the immunoglobulin class and subclass content of 12 inhibitors with monoclonal antibodies specific for human IgG subclasses and IgM. Seven hemophilic (alloantibody) and five spontaneous (autoantibody) inhibitors contained IgG-1 and IgG-4 antibody; one of the spontaneous inhibitors also contained IgG-3. In one hemophilic inhibitor, the IgG-4 component reacted strongly with the 92-kd and 80-kd polypeptides, whereas the IgG-1 component reacted only minimally with the 92-kd polypeptide although its reactivity with the 80-kd polypeptide was strong. Another hemophilic inhibitor was affinity purified and subjected to quantitative radial immunodiffusion, and the presence of IgG-1 and IgG-4 antibody was confirmed. We conclude that the inhibitors examined are not monoclonal but are probably of restricted polyclonal origin and that different IgG subclasses in an inhibitor plasma can have different degrees of FVIII polypeptide reactivity.

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THE FVIII coagulant protein (FVIII) is a cofactor protein that promotes the enzymatic activation of factor X by activated factor IX in the intrinsic blood coagulation pathway. FVIII circulates in blood as a complex with von Willebrand factor, a multimeric glycoprotein involved in platelet adhesion. The FVIII protein is absent or defective in individuals with hemophilia A, an X-linked, recessive bleeding disorder with a frequency of ~1 in 10,000 in the general population. Gene cloning data have shown that FVIII is a single-chain protein with a calculated mol wt of 264,763.[1] When the protein is purified from plasma, however, multiple FVIII fragments are obtained, due to its extreme sensitivity to proteolysis by thrombin and other enzymes during purification.[1-9]

FVIII inhibitors are antibodies that neutralize the coagulant activity of FVIII. Inhibitors arise as alloantibodies in ~8% to 20% of individuals with severe hemophilia A in response to infusion of FVIII concentrates.[10] Inhibitors can also arise spontaneously as autoantibodies either in normal individuals, or in those with certain other conditions, including immunologic disorders.[10] The underlying basis for inhibitor development is unknown, and no entirely satisfactory approach to treatment currently exists.

Many investigators have attempted to analyze the immunoglobulin content of FVIII inhibitors with immunoneutralization assays based on use of absorbed polyclonal antisera against human immunoglobulin classes and subclasses to neutralize the anti-FVIII activity of inhibitor antibodies in a coagulation assay. Several studies involved five or more patient samples in which both heavy and light chains were typed.[11-14] These and other data were summarized by Hoyer and colleagues[15] as follows. Most inhibitors were IgG, although a few IgM inhibitors were reported. Among the IgG subclasses, most inhibitors were IgG-4, often with at least one other heavy chain subclass. A few inhibitors were reported to be IgG-3 only, but none were only IgG-1 or IgG-2. The inhibitors contained either κ or λ light chains or both. Based on these data, most inhibitors were believed to be of a restricted polyclonal origin.

In the studies reported here, immunoblotting of FVIII with the inhibitor antibodies and the use of monoclonal anti-immunoglobulin antibodies allowed a direct, unambiguous demonstration of at least two immunoglobulin types in each inhibitor. In addition, differences between FVIII polypeptide reactivity and IgG subclass were demonstrated in one individual. Although the immunoblotting technique is not quantitative and only inhibitor epitopes that are not destroyed in the immunoblotting process can be analyzed, it provides data important in the biochemical characterization of FVIII inhibitor antibodies and in the study of the immune response.

MATERIALS AND METHODS

FVIII purification and digestion with thrombin. FVIII was purified from commercial FVIII concentrate by immunoadsorbent chromatography[6,16,17] and digested with thrombin[18] as previously described.

Inhibitor assay. Inhibition of FVIII coagulant activity by inhibitor antibodies was measured in Bethesda units (BU) by the method of Kasper and co-workers.[19]

Electrophoresis. Samples of purified FVIII were dialyzed against sample buffer containing 0.01 mol/L of sodium phosphate, 1% $NaDodSO_4$ and 10 mmol/L of disodium EDTA, pH 7.0, overnight at room temperature. Discontinuous $NaDodSO_4$ 7.5% polyacrylamide slab gel electrophoresis was performed as previously described.[6] The dialyzed FVIII was applied to the gel at ~4 μg per lane, without prior heating or reduction.

Immunoblotting. Separated FVIII fragments were electrophoretically transferred onto nitrocellulose membranes as previously described,[17] except that 0.1% $NaDodSO_4$ was added to the transfer buffer.

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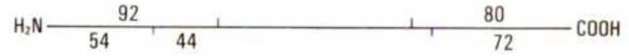

Fig 1. Line diagram of a single-chain FVIII molecule with enzyme cleavage sites indicated above and below. Top: Purified FVIII is isolated as a series of polypeptides of 80 kd, 92 kd, and multiple higher mol wt polypeptides representing the amino terminal 92-kd portion with various carboxyl terminal cleavages into the midsection of the molecule. Bottom: Thrombin produces the 92-kd polypeptide and further cleaves it to 54 kd and 44 kd. Thrombin also produces the 72-kd fragment from its 80-kd precursor. The enzyme giving rise to the 80-kd polypeptide has not been identified. These data are from published reports of FVIII gene cloning and protein biochemistry.[1,2,5]

Processing of immunoblots. Nitrocellulose membranes containing transferred FVIII fragments were incubated for 6 hours in 100 mL of Blotto buffer [phosphate-buffered saline (PBS) containing 5% nonfat dry milk] prepared according to Johnson and colleagues.[20] All incubations were done on a rotary shaker at room temperature. The membranes were then incubated overnight in 100 mL of Blotto buffer containing plasma samples (up to 2 mL, depending on titer) and protease inhibitors, both prepared as previously described.[18]

After incubation with plasma samples, the membranes were washed in 100 mL of PBS containing 0.05% Nonidet P-40 for 30 minutes, with changes of buffer at 10 and 20 minutes. They were subsequently rinsed in Blotto buffer and then incubated for 2 hours in 100 mL of Blotto buffer with 10 μL of ascites fluid containing monoclonal anti-human IgG-1, IgG-2, IgG-3, IgG-4, anti-κ light chains, anti-λ light chains, or anti-IgM antibodies (ICN Immuno-Biologicals, Lisle, IL). The monospecificity of these monoclonal anti-human immunoglobulin class and subclass antibodies has been determined by the laboratories of Miles-Yeda, Rehovat, Israel, using an enzyme-linked immunosorbent assay (ELISA) assay with freshly prepared antigens. Membranes were then washed for 30 minutes, as above, in Blotto buffer.

The FVIII/inhibitor antibody/monoclonal anti-immunoglobulin antibody complexes were then visualized by incubation of the membranes for at least 30 minutes in 100 mL of Blotto buffer containing ^{125}I-labeled, affinity-purified rabbit anti-mouse immunoglobulin antibody, followed by washing in Blotto buffer as above. After membranes were briefly rinsed in PBS, autoradiography was performed at −70°C as previously described.[18] Film exposure times are indicated in Figs 1 through 3.

Negative controls for the immunoblots consisted of electrophoresing 2.5 μg of bovine serum albumin (BSA) in a lane adjacent to FVIII. Positive controls that demonstrated the reactivity of the monoclonal anti-human immunoglobulin antibodies with human immunoglobulins under these conditions consisted of substituting affinity-purified goat anti-human IgG, IgA, and IgM antibody for purified FVIII, as well as substituting normal human plasma for inhibitor plasma. Thus, immobilized goat anti-human immunoglobulin antibody was used to trap normal human immunoglobulins. This procedure was necessary because some of the monoclonal anti-human immunoglobulin antibodies did not react well with human immunoglobulins that were immobilized directly on nitrocellulose.

Affinity purification of inhibitor antibody. Inhibitor antibody was purified directly from plasma by passage over a column of FVIII-Sepharose. The column was prepared by coupling 1 mg (2,000 U) of partially purified FVIII to ~1 mL of cyanogen bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. A 0.5-mL sample of inhibitor plasma was diluted to 4 mL in 0.02 mol/L of imidazole, 0.15 mol/L of sodium chloride, pH 7.0, containing protease inhibi-

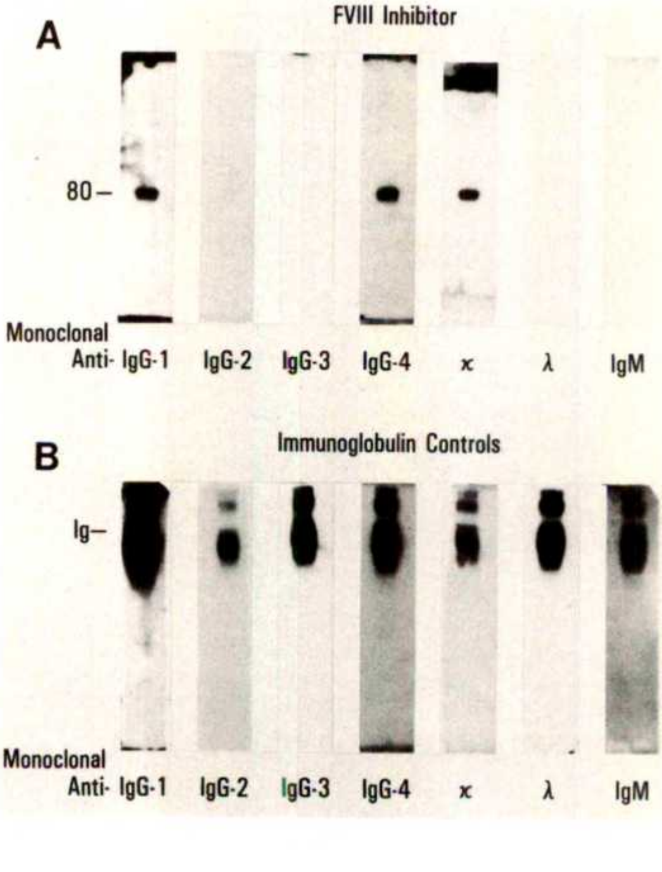

Fig 2. Analysis of the IgG subclass and IgM content of a FVIII inhibitor (A in Table 2) as described in the Materials and Methods section. (A) Autoradiographs showing the reactivity of the 80-kd FVIII polypeptide with monoclonal anti-IgG-1, anti-IgG-4, and anti-κ light chain antibodies and its lack of reactivity with monoclonal anti-IgG-2, IgG-3, λ light chain and IgM antibodies. Film exposures were, from left to right, 4, 7, 7, 4, 3, 7, and 2.5 days. (B) Autoradiographs showing the positive control corresponding to each immunoblot in the top panel as described in the Materials and Methods section. Film exposures were the same as above.

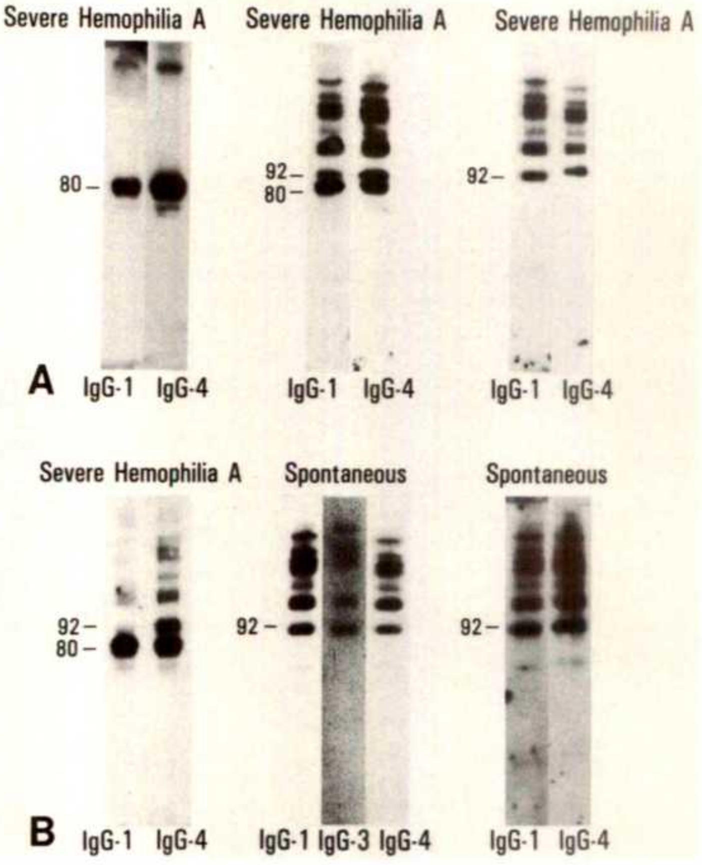

Fig 3. Immunoblots of four hemophilic and two spontaneous FVIII inhibitors from Table 2 showing positive reactions for IgG-1, IgG-4, and (in one case) a weakly positive reaction for IgG-3. The 80-kd carboxyl terminal and 92-kd amino terminal polypeptides of FVIII are indicated (as in Fig 1). Inhibitors (described in Table 2 A, G, D, F, H, and I) and film exposure times are as follows: (A) Left to right: plasma A IgG-1 2 days and IgG-4 7 days (as in Fig 2); plasma G IgG-1 7 days and IgG-4 8 days; plasma D IgG-1 7 days and IgG-4 2 days. (B) Left to right: plasma F IgG-1 7 days and IgG-4 7 days; plasma H IgG-1 7 days, IgG-3 14 days, and IgG-4 8 days; and plasma I IgG-1 8 days and IgG-4 7 days.

tors[18] and passed repeatedly over the column until 89% of the inhibitor activity (in BU) was adsorbed.

After extensive washing of the column with the above buffer, inhibitor antibody was desorbed by two successive elutions with each of the following eluants: 3 mol/L of sodium thiocyanate in 0.05 mol/L of sodium acetate, 0.1 mol/L of lysine, pH 5.5; 0.2 mol/L of HCl adjusted to pH 2.2 with glycine; and 0.05 mol/L of diethylamine, pH 11. Eluates were neutralized and then both dialyzed and concentrated on a Centricon 30 membrane (Amicon, Danvers, MA). Concentrated samples were then assayed for inhibitory activity (BU) and IgG content (see below).

Quantitation of affinity-purified inhibitor IgG content by radial immunodiffusion. Commercially available radial immunodiffusion (RID) plates (ICN Immunobiologicals) were used to quantitate the IgG-1, IgG-2, IgG-3, and IgG-4 content of affinity-purified inhibitor antibody. The manufacturer's instructions were followed, except that the affinity-purified inhibitor (in buffer) was diluted with an equal volume of twofold concentrated sheep serum (provided by the manufacturer in lyophilized form), to maintain comparability with the human serum standards which were diluted in sheep serum.

RESULTS

FVIII polypeptide specificity of FVIII inhibitors as determined by immunoblotting. In a previous report,[18] we used immunoblotting to localize FVIII inhibitor epitopes in 25 FVIII inhibitor patients to either the 72-kd thrombin fragment of FVIII or the 44-kd thrombin fragment or both of these fragments. These two fragments are derived from the 80-kd and 92-kd precursor polypeptides, respectively (Fig 1). We extended our study to include 76 hemophilic and spontaneous FVIII inhibitor patients, and the results are presented in Table 1. In all but two patients, the FVIII inhibitor epitopes were localized to either or both the 72-kd and 44-kd thrombin fragments. The two exceptions were hemophilic inhibitors with epitopes on both the 54-kd and 44-kd thrombin fragments. These results extend the preliminary FVIII inhibitor epitope map[18] to include all fragments believed to be important to FVIII procoagulant activity.[2-4,16,17,21-24] Most FVIII inhibitor epitopes, however, were restricted to the 72-kd and 44-kd thrombin fragments.

Due to the smaller number of spontaneous FVIII inhibitor samples tested, we cannot yet draw conclusions about possible differences in the distribution of FVIII fragment specificities between spontaneous and hemophilic FVIII inhibitors. Also, as indicated in Table 1, some immunoblots gave inconclusive data due to very weakly reactive inhibitors. This

Table 1. Immunoblotting Analysis of FVIII Polypeptide Specificity of 54 Hemophilic and 22 Spontaneous FVIII Inhibitors

Inhibitor	72 kd	44 kd	72 kd and 44 kd	54 kd and 44 kd	Inconclusive	Total
Hemophilic	24	5	16	2	7	54
Spontaneous	9	6	6	0	3	22

Molecular weights (kd) of FVIII thrombin fragments containing inhibitor epitopes.

weak reactivity could result from loss of conformationally dependent epitopes during the immunoblotting process, low antibody titer, low-affinity antibodies, or technical reasons. In some cases, repeating the immunoblots was sufficient to resolve the uncertainty. In addition, 14 of the inhibitor plasmas we analyzed were identified by Hoyer and coworkers[15] as being either type I or type II, based on their kinetics of FVIII inactivation. Type I inhibitors completely inactivate FVIII activity, whereas type II inhibitors do not.[15] Among the ten type I and four type II inhibitors we studied, there was no clear correlation between type I or type II behavior and FVIII fragment specificity.

Determination of inhibitor class and subclass by immunoblotting. An example of the use of immunoblotting to analyze the immunoglobulin class and subclass composition of FVIII inhibitors is shown in Fig 2. This inhibitor (A in Table 2) was a high-titer inhibitor with epitopes on the 80-kd FVIII polypeptide (and its 72-kd thrombin fragment). It was positive for IgG-1, IgG-4, and κ light chains. Although the inhibitor was also weakly positive for IgG-2, IgG-3, and λ light chains, this was not apparent after multiple photographic reproductions. Due to the very weak nature of the reactions, these latter immunoblots were considered inconclusive. The inhibitor was negative for IgM. Also shown in Fig 2 are the positive controls used in these experiments, indicating that all monoclonal anti-immunoglobulin antibodies were reactive with human immunoglobulins in this system. The monoclonal anti-IgG-2 antibody showed a weaker than expected positive control, however (based on the relative amount of IgG-2 in plasma), and, in most subsequent immunoblots, the IgG-2 positive control was too weak to yield interpretable results. In addition, several inhibitors tested negative for both κ and λ light chains, resulting in a lack of reliable data for light chain typing. It is possible that light chain variants exist that lack the epitopes for these monoclonal antibodies. Therefore, immunoglobulin class and subclass determination using this technique was largely limited to the heavy chains of IgG-1, IgG-3, IgG-4, and IgM.

Table 2. IgG Subclass Content of 12 FVIII Inhibitor Plasmas as Determined by Immunoblotting

Inhibitor	Inhibitor Type	FVIII Fragment (kd)	Titer (BU/mL)	IgG-1	IgG-2	IgG-3	IgG-4	IgM
A	HA	80	7,800	Pos	—	—	Pos	Neg
B	HA	80	167	Pos	Neg	Neg	Pos	Neg
C	HA	92	5.3	Pos	—	Neg	Pos	Neg
D	HA	92	135	Pos	—	Neg	Pos	Neg
E	HA	92, 80	150	Pos	Neg	Neg	Pos	Neg
F	HA	92, 80	375	Pos	—	Neg	Pos	Neg
G	HA	92, 80	3,000	Pos	—	Neg	Pos	ND
H	SP	92	3,600	Pos	—	Pos	Pos	ND
I	SP	92	500	Pos	—	Neg	Pos	ND
J	SP	92	58	Pos	—	Neg	Pos	Neg
K	SP	80	1,700	Pos	—	Neg	Pos	ND
L	SP	80	960	Pos	—	Neg	Pos	ND

HA, hemophilia A; SP, spontaneous; Pos, positive; Neg, negative; —, inconclusive; ND, not determined; BU, Bethesda units.

IgG subclass composition and FVIII polypeptide specificity of seven hemophilic and five spontaneous FVIII inhibitors as determined by immunoblotting. Table 2 and Fig 3 summarize data on the IgG subclass composition of 12 FVIII inhibitors using this technique. Seven inhibitors were from severe, multitransfused hemophilia A patients and five were of the spontaneous type. The data in Table 2 show that all 12 inhibitors were positive for both IgG-1 and IgG-4 antibodies. Of seven tested, none was positive for IgM. All except one of the inhibitors were negative for IgG-3. The exception was a spontaneously arising inhibitor (H in Table 2), that showed a weak, but positive reaction with the monoclonal anti-IgG-3 antibody (Fig 3). As noted above, most data for IgG-2 were inconclusive.

The IgG-1 and IgG-4 components of inhibitor F showed differences in the degree of reactivity with some of the FVIII polypeptides when immunoblotted and autoradiographed simultaneously (Fig 3). Although the IgG-1 component was strongly reactive with the 80-kd FVIII polypeptide, it reacted only minimally with the 92-kd polypeptide (and its higher mol wt precursors). In contrast, the IgG-4 component was strongly reactive with all polypeptides. This is the first evidence for differences between FVIII polypeptide reactivity and IgG subclass.

Affinity purification of a hemophilic FVIII inhibitor and quantitation of the IgG subclass content by radial immunodiffusion. Inhibitor G in Table 2 was affinity purified from plasma by a one-step adsorption onto FVIII-Sepharose as described in the Materials and Methods section. Of 1,622 BU applied to the column, 1,440 BU were bound, and 240 BU (17% of bound) were recovered in the final concentrated pool. The IgG-1, IgG-2, IgG-3, and IgG-4 contents of the affinity-purified inhibitor were quantitated by RID against monospecific polyclonal anti-human IgG subclass antisera, as described in the Materials and Methods section. The affinity-purified inhibitor sample contained 8.8 μg of IgG-1 and 11.6 μg of IgG-4. No IgG-3 was found and, as with the immunoblotting system, the results for IgG-2 were inconclusive due to weak positive controls. The quantitative RID results therefore confirmed the qualitative immunoblotting results, which showed that the inhibitor contained both IgG-1 and IgG-4 components.

DISCUSSION

Hoyer and colleagues[15] noted the predominance of IgG-4 in FVIII inhibitors, even though IgG-4 constitutes only ~4% of total serum IgG. Although the reason for this is not known, Hoyer and colleagues note that a predominantly IgG-4 response has been hypothesized by Aalberse and co-workers[25] to be associated with chronic antigenic stimulation. Aalberse and co-workers showed that in novice beekeepers, the antibody response to the phospholipase A2 of bee venom shifts over time from an IgG-1 dominated response to an IgG-4 dominated response following repeated bee stings. These data provide an attractive hypothesis that FVIII inhibitors, particularly in multitransfused hemophiliacs, would be characterized by an IgG-4–dominated response. Quantitation of IgG-1 and IgG-4 in patient G by

RID showed them to be present in nearly equal amounts. However, the low (17%) yield of purified inhibitor IgG raises the possibility that only a selected population of the total inhibitor content may have been isolated, and it may not represent the actual relative amounts of IgG subclases present.

Previous FVIII inhibitor subtyping data based on immunoneutralization assays compiled from many laboratories suggested that approximately half of FVIII inhibitors were IgG-4 only and half were IgG-4 plus at least one other heavy chain subclass.[15] In only one report[12] did six of seven inhibitors tested by immunoneutralization assay contain both IgG-1 and IgG-4. The immunoblotting data presented here clearly show that in each of the 12 inhibitor plasmas tested, both IgG-1 and IgG-4 antibody were present, suggesting that the occurrence of IgG-1 antibody in FVIII inhibitors may be a more general phenomenon than previously thought.

The immunoblotting data also show that in each of the 12 inhibitor plasmas tested, the restriction of FVIII epitopes to the 80-kd FVIII polypeptide, the 92-kd polypeptide, or both of these polypeptides was maintained by each IgG subclass analyzed. Analysis of inhibitor F, however, showed that differences in FVIII polypeptide reactivity between IgG subclasses in the same individual are possible. In this inhibitor both the IgG-1 and IgG-4 components reacted equally well with the 80-kd polypeptide, whereas the IgG-4 component reacted much more strongly with the 92-kd polypeptide than did the IgG-1 component.

The spontaneous, autoantibody inhibitors showed an IgG subclass composition similar to that of hemophilic alloantibodies, although the only inhibitor found to contain IgG-3 in addition to IgG-1 and IgG-4 was of the spontaneous type. It seems likely that, as previously suggested,[15] FVIII inhibitors are of a restricted polyclonal, or oligoclonal origin. The present data, however, cannot rule out the possibility that some of these inhibitors might be biclonal (composed of two monoclonal antibodies) in origin. Further investigation of the number of FVIII epitopes for these inhibitors should help to answer this question.

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Protein C Inhibits Endocytosis of Thrombin–Thrombomodulin Complexes in A549 Lung Cancer Cells and Human Umbilical Vein Endothelial Cells

By Ikuro Maruyama and Philip W. Majerus

We investigated the effect of protein C on the endocytosis of thrombin–thrombomodulin complexes. We previously showed that exposure of umbilical vein endothelial cells to thrombin stimulated the internalization and degradation of thrombin. A similar internalization was stimulated by a monoclonal antithrombomodulin antibody. We have repeated these studies in the presence of protein C and found that endocytosis of ^{125}I-thrombin–thrombomodulin complexes, but not ^{125}I-antithrombomodulin–thrombomodulin complexes, is inhibited. Activated protein C did not inhibit endocytosis of thrombin–thrombomodulin complexes. Protein C inhibited both internalization and degradation of ^{125}I-thrombin and diisopropylphosphoryl (DIP) ^{125}I-thrombin in human lung cancer cells (A549). These effects were observed at protein C concentrations found in human plasma. Protein S had no effect on the inhibition of endocytosis of thrombin–thrombomodulin complexes by protein C. We propose that protein C may regulate the rate of endocytosis of thrombin–thrombomodulin complexes in vivo and thereby control the capacity for endothelium to activate protein C.

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RAPID PROTEIN C activation by thrombin depends on the endothelial cell surface protein, thrombomodulin.[1,2] Thrombin binds to thrombomodulin in a 1:1 complex that catalyzes protein C activation.[3,4] Previously, we found that exposure of cultured human umbilical vein endothelial cells or A549 human lung cancer cells to thrombin decreased thrombomodulin on the cell surface, suggesting that internalization of thrombin–thrombomodulin had occurred. The internalized ^{125}I-thrombin was degraded in the cells, and thrombomodulin reappeared on the cell surface after 30 minutes, implying the recycling of thrombomodulin.[5] From these observations, we proposed that the uptake and degradation of thrombin bound to thrombomodulin on the endothelial cell surface may stop protein C activation and provide a mechanism for clearance of thrombin from the circulation. Our previous results indicated that within 30 minutes of exposure to thrombin, all active thrombin–thrombomodulin complexes were cleared from the surface of A549 cells. This result was in apparent conflict with the original study of Esmon and Owen[1] who showed a linear rate of protein C activation for at least 1 hour when thrombin and protein C were incubated with human umbilical vein endothelial cells. We studied endocytosis of thrombin–thrombomodulin complexes in the presence of protein C to resolve this apparent conflict, and found that protein C, but not activated protein C, inhibits the endocytosis of thrombin–thrombomodulin complexes both in human umbilical vein endothelial cell cultures and in A549 cell cultures.

MATERIALS AND METHODS

Materials were obtained from the following sources: Medium 199 (M199) and fetal calf serum (FCS) from KC Biological (Lenexa, KS); penicillin, streptomycin, and L-glutamine from GIBCO Laboratories (Grand Island, NY); carrier-free ^{125}I-sodium iodide from Amersham (Arlington Heights, IL); tissue culture flasks and centrifuge tubes from Corning Glass Works (Science Products, Corning, NY); and Falcon Labware (Becton Dickinson, Oxnard, CA); Cluster 24-, 48-dish wells from Costar (Cambridge, MA); and protein A-Sepharose from Pharmacia Fine Chemicals (Piscataway, NJ). All other chemicals were reagent grade products of Sigma Chemical (St Louis) or Fisher Scientific (Pittsburgh). Protein C[6] and thrombin[7] were of human origin and isolated as indicated. Protein S from human plasma[8] was provided by Dr Koji Suzuki (Mie University School of Medicine, Tsu, Japan). The proteins were homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (BSA) as standard. Monoclonal antithrombomodulin antibody was produced as described previously.[5] Antithrombomodulin monoclonal IgG (designated IgG–TM) was isolated from ascites using protein A-Sepharose.[5] Protein C was fully activated by thrombin, and the activated protein was subsequently isolated.[8]

Protein iodination. IgG–TM (7,000 to 20,000 cpm/ng) and thrombin (1,000 to 2,000 cpm/ng) were labeled with ^{125}I-iodine by a modified chloramine T procedure.[5] Protein was separated from free iodide on a Sephadex G-25 column. The specific activity in a coagulation assay of the α-thrombin used was ~2,200 NIH U/mg of protein. Iodinated thrombin was treated with diisopropyl fluorophosphate (DFP) as described previously.[5] Unreacted DFP was removed by gel filtration on a column of Sephadex G-25.

Cell cultures. Human umbilical vein endothelial cells were prepared by the method of Jaffe and co-workers.[9] A human lung cancer (A549) cell line was obtained from the American Type Culture Collection. Cells were grown in 6- to 16-mm multiwell plates using M199 containing 20% FCS, 2 mmol/L of glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a 5% CO_2 atmosphere at 37°C. Cell number was determined using a Coulter Counter model F (Coulter Electronics, Hialeah, FL).[10]

Binding studies. Confluent monolayers of human umbilical vein endothelial cells in 10-mm culture wells were washed three times with 0.14 mol/L of NaCl, 0.004 mol/L of KCl, 0.001 mol/L of phosphate buffer, pH 7.4, 0.011 mol/L of glucose containing 0.2% BSA (henceforth referred to as wash buffer). Binding studies were carried out in reaction mixtures containing M199 supplemented

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with 2.5 mg/mL of ovalbumin and 10 mmol/L of Hepes, pH 7.4 (thrombin-binding medium) for ^{125}I-thrombin binding, or M199 containing 5 mg/mL of BSA and 50 μg/mL normal human IgG (IgG–TM binding buffer) for ^{125}I-IgG–TM binding in a total volume of 0.3 mL. After incubation of cells with ^{125}I-labeled protein, the cells were washed rapidly five times with cold (0°C) wash buffer. The washed monolayers were solubilized in 0.5 mL of buffer containing 15% glycerol, 2% sodium dodecyl sulfate (SDS), 75 mmol/L of Tris-HCl (pH 6.8), and 2 mmol/L of EDTA (cell-solubilizing buffer). The solubilized cell solution was assayed for radioactivity in a Biogamma II scintillation counter (Beckman Instruments, Fullerton, CA). Specific binding was determined by measuring the difference in cell-bound radioactivity with and without 100-fold excess unlabeled ligand. The cell-bound radioactivity with an excess amount of unlabeled ligand was considered nonspecific and was subtracted from the total binding. Nonspecific binding was 20% to 40% of total binding using ^{125}I-thrombin and 10% to 25% of total binding using ^{125}I-IgG–TM. In experiments in which endocytosis and degradation of thrombin were measured, bound thrombin was estimated as that displaced by unlabeled thrombin, degraded thrombin was estimated as TCA soluble radioactivity in the medium, and internalized thrombin was that not displaced by unlabeled thrombin after 60 minutes at 4°C.

RESULTS

Inhibition by protein C of the endocytosis of thrombin– or diisopropylphosphoryl (DIP)–thrombin–thrombomodulin complexes in human umbilical vein endothelial cells. We previously showed that thrombomodulin disappears from the surface of human umbilical vein endothelial cells after 5-minute exposure to thrombin. Cell surface thrombomodulin was estimated by measuring the binding of ^{125}I-IgG–TM at various times after exposure of cells to thrombin. We repeated this experiment with and without protein C, as shown in Fig 1. Exposure of human umbilical vein endothelial cells to thrombin or DIP–thrombin for 5 minutes resulted in a 50% decrease in cell surface thrombomodulin at 45 minutes, with some return of thrombomodulin to the cell surface by 65 minutes. With 0.12 μmol/L of protein C, however, the decrease in cell surface thrombomodulin did not occur. The inhibition of endocytosis was similar using either active or inactive (DIP) thrombin, suggesting that protein C activation was not required to produce this effect. The results shown are from one of five similar experiments. Next, we repeated this experiment comparing the effect of activated protein C to protein C; only the latter inhibited endocytosis of thrombomodulin, as shown in Table 1. The effect of varying protein C concentration on the inhibition of endocytosis is also shown in Table 1. We achieved maximal inhibition at 0.12 μmol/L of protein C, which is approximately twice the plasma concentration of this factor in humans. In other experiments, we found no greater inhibition of endocytosis at protein C concentrations of up to 2 μmol/L. Similarly, activated protein C was tested over a range of 0 to 2 μmol/L without effect in 14 experiments. We also added protein S at 1 to 4 μmol/L with varying concentrations of protein C (0 to 0.06 μmol/L) to determine whether this protein would modify the ability of protein C to inhibit endocytosis. No effect of protein S was observed.

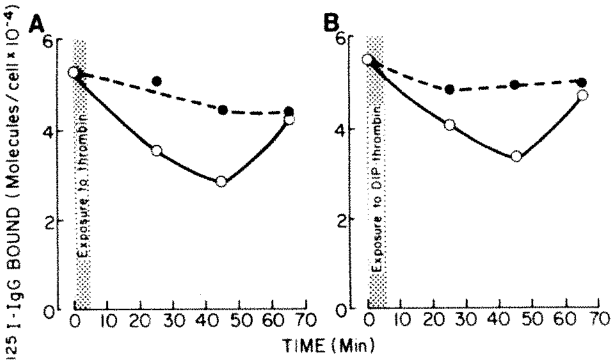

Fig 1. Inhibition of the endocytosis of thrombin- (A), or diisopropylphosphoryl (DIP)–thrombin–thrombomodulin complex (B) in human umbilical vein endothelial cells. Monolayers of human umbilical vein endothelial cells were washed three times with wash buffer, then exposed to 8 nmol/L of unlabeled thrombin or DIP–thrombin for 5 minutes at 37°C. The monolayers were washed once with wash buffer at 0°C, and ^{125}I-IgG–TM binding medium was added with (●—●) or without (○—○) 0.12 μmol/L of protein C. At the times indicated, ^{125}I-IgG–TM (17 nmol/L) was added and after a 5-minute incubation at 37° bound ^{125}I-IgG–TM (15,000 cpm/ng) was determined as described in the Materials and Methods section. Nonspecific binding was 6,000 cpm/well; total binding before exposure to thrombin was 37,000 and 36,000 cpm/well.

Effect of protein C or activated protein C on IgG–TM-induced decrease of thrombomodulin molecules on human umbilical vein endothelial cells. Monoclonal anti-thrombomodulin IgG, designated IgG–TM, also induces a decrease in thrombomodulin molecules on endothelial cells.[5] As shown in Fig 2, exposure of human umbilical vein endothelial cells to IgG–TM resulted in the reduction of thrombomodulin molecules on the cell surface. The decrease in surface thrombomodulin was greater than that induced by thrombin or DIP–thrombin. Reappearance of thrombomod-

Table 1. Effect of Protein C or Activated Protein C on Thrombin-Induced Endocytosis of Thrombomodulin in Human Umbilical Vein Endothelial Cells

Agent	Thrombomodulin (%)*
Protein C (μmol/L)	
0	60
0.03	70
0.06	77
0.12	99
Activated protein C	
0.06	61
0.12	63

Confluent monolayers of human umbilical vein endothelial cells (1 × 10^5) were washed three times with wash buffer and were then exposed to 8 nmol/L of unlabeled thrombin for 5 minutes at 37°C. The monolayers were washed once with cold wash buffer, and ^{125}I-IgG–TM binding medium containing 0 to 0.12 μmol/L of protein C or activated protein C was added and incubated another 15 minutes at 37°C. Then the monolayers were incubated with ^{125}I-IgG–TM (15,000 cpm/ng) (11 nmol/L) for 60 minutes at 4°C, and bound ^{125}I-IgG–TM was determined.

*The 100% control value for cell surface thrombomodulin (thrombomodulin molecules on the cell surface before exposure to thrombin) was 44,000 molecules per cell.

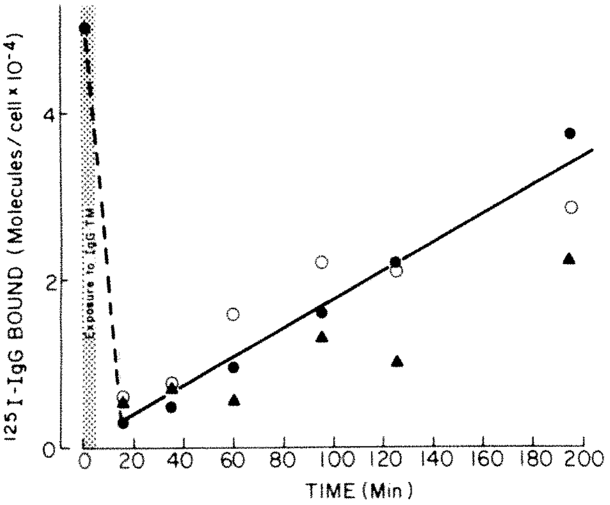

Fig 2. Thrombomodulin endocytosis on human umbilical vein endothelial cells after exposure to IgG–TM. Monolayers of human umbilical vein endothelial cells were washed three times with wash buffer, then exposed to 25 nmol/L of unlabeled IgG–TM for 5 minutes at 37°C. The monolayers were washed once with wash buffer at 0°C, and ^{125}I–IgG–TM binding medium was added with (○) or without (●) 0.1 μmol/L of protein C or activated protein C (▲). At times indicated, ^{125}I-IgG–TM (25 nmol/L) was added, and after a 5-minute incubation at 37°C, bound ^{125}I-IgG–TM (7,500 cpm/ng) was determined. Nonspecific binding in this experiment was 6,500 cpm. Total bound before exposure to unlabeled IgG–TM was 23,000 cpm/well.

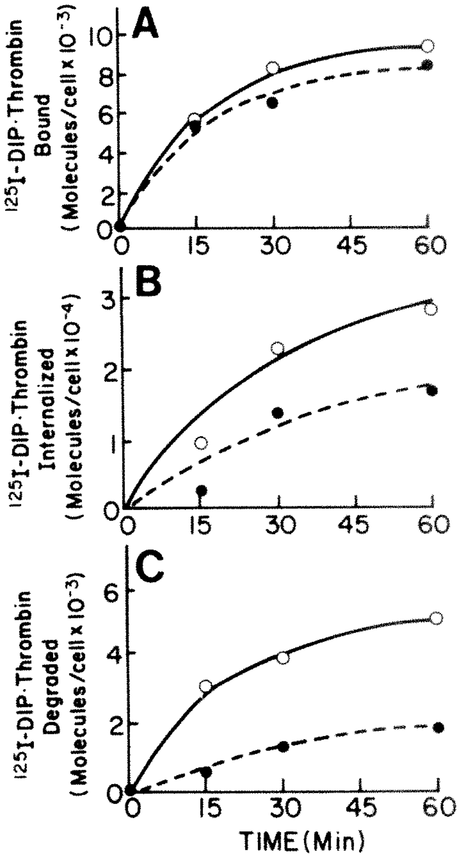

Fig 3. Binding, endocytosis, and degradation of ^{125}I-diisopropylphosphoryl (DIP)–thrombin in A549 cells at 37°C with or without protein C. Monolayers of A549 cells were incubated with thrombin binding medium (300 μL) containing ^{125}I-DIP–thrombin (8 nmol/L) with or without 0.12 μmol/L of protein C at 37°C. At the times indicated, the monolayers were washed with cold wash buffer, and fresh thrombin binding medium (300 μL) containing 100 nmol/L of unlabeled DIP–thrombin was added. The monolayers were incubated at 4°C for an additional 60 minutes. After each incubation period, the total amount of surface ^{125}I-DIP–thrombin (A), internalized ^{125}I-DIP–thrombin (B), and ^{125}I-DIP–thrombin degradation products in the medium (C) was determined as described in the Materials and Methods section. ○—○, minus protein C; ●—●, with protein C.

ulin molecules on the cell surface was also delayed and occurred over 3 hours. Neither protein C nor activated protein C inhibited IgG–TM-induced endocytosis of thrombomodulin. This experiment was performed six times with similar results. These results suggest that protein C only inhibits endocytosis of the thrombin–thrombomodulin complex.

Effect of protein C on the time course of binding, internalization, and degradation of ^{125}I-DIP–thrombin in A549 cells. We investigated the effect of protein C on the time course of binding, internalization, and degradation of ^{125}I-DIP-thrombin in A549 Cells at 37°C, as shown in Fig 3. At each incubation period, monolayers were washed with cold wash buffer, and fresh thrombin-binding medium (300 μL) containing 100 nmol/L of unlabeled DIP–thrombin was added and incubated at 4°C for an additional 60 minutes. TCA soluble radioactivity from the first incubation medium reflects degraded ^{125}I-DIP–thrombin, and displaced radioactivity by unlabeled excess DIP–thrombin in the second incubation reflects cell surface bound ^{125}I-DIP–thrombin. As shown in the top panel of Fig 3, there was no significant difference in surface-bound ^{125}I-DIP–thrombin in experiments with or without protein C. ^{125}I-DIP–thrombin degradation (middle panel) and ^{125}I-DIP–thrombin internalization (bottom panel), however, were significantly inhibited by the addition of protein C. Next, we investigated the inhibition of endocytosis and degradation of ^{125}I-DIP–thrombin at varying protein concentrations, as shown in Fig 4. In this experiment, A549 cell monolayers were incubated with or without various amounts of protein C and 2.5 nmol/L of ^{125}I-DIP–thrombin for 4 hours. Protein C inhibited endocytosis and degradation of ^{125}I-DIP–thrombin but not the binding to the cell surface. There was 40% to 70% inhibition of endocytosis and degradation at the highest concentration of protein C used.

DISCUSSION

In this study, we investigated the effect of protein C or activated protein C on thrombin-, DIP–thrombin-, or IgG–TM-induced endocytosis of thrombomodulin in cultured human umbilical vein endothelial cells and in A549 human lung cancer cells. Protein C, but not activated protein C, at physiological concentrations inhibited thrombin or DIP–thrombin-induced endocytosis of thrombomodulin and subsequent degradation of thrombin or DIP–thrombin on human umbilical vein endothelial and A549 cells. IgG–TM also induced endocytosis of cell-surface thrombomodulin in

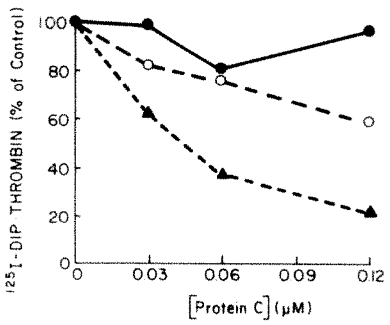

Fig 4. Inhibition of endocytosis and degradation of ^{125}I-diisopropylphosphoryl (DIP)-thrombin in A549 cells at 37°C by increasing amounts of protein C. Monolayers of A549 cells were incubated with thrombin binding medium (300 μL) containing the indicated concentration of protein C and 8 nmol/L of ^{125}I-DIP-thrombin (2,000 cpm/ng) at 37°C. After 4 hours of incubation, medium was removed by washing three times with cold wash buffer, and fresh thrombin binding medium (300 μL) containing 100 nmol/L of unlabeled DIP-thrombin was added. The monolayers were incubated at 4°C for an additional 60 minutes, after which the total amount of surface ^{125}I-DIP-thrombin (●), internalized ^{125}I-DIP-thrombin (○), and ^{125}I-DIP-thrombin degradation products in the original medium (▲) were determined as described in the Materials and Methods section. The 100% control values for surface-bound, internalized, degraded ^{125}I-DIP-thrombin in the absence of protein C were 11,000, 15,500, and 9,000 molecules per cell, respectively.

human umbilical vein endothelial cells and A549 cells, but protein C did not inhibit this reaction. Protein S, a potentiator of the activity of activated protein C in the inactivation of coagulation factors Va and VIIIa,[8,11] had no effect on the protein C inhibition of endocytosis of thrombomodulin. The physiological significance of the finding that protein C inhibits thrombin-induced endocytosis of thrombomodulin in vitro remains uncertain. On the basis of our findings, we propose that protein C in situ may serve to regulate thrombin-induced endocytosis of thrombomodulin. When thrombin is formed in intravascular sites near endothelium, the subsequent formation of thrombin–thrombomodulin complex on endothelial cells tends to inhibit procoagulant reactions of thrombin[3] and lead to protein C activation. The activated protein C formed may then combine with protein S, bind to another site on endothelium where factors Va and VIIIa are inactivated, and thereby inhibit fibrin formation and act as a local anticoagulant.[12] Once protein C is depleted locally, endocytosis of thrombin–thrombomodulin complex and subsequent degradation of thrombin may occur. Thus, endocytosis of thrombin–thrombomodulin and regulation of this process by protein C may provide a control mechanism for the thrombin–thrombomodulin system on the endothelial cell surface. The recent discovery that bovine thrombomodulin is similar in structure to receptors that undergo endocytosis in coated pits lends additional credence to the hypothesis proposed here.[13]

ACKNOWLEDGMENT

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Binding and Internalization of Recombinant Human Erythropoietin in Murine Erythroid Precursor Cells

By R.A. Mufson and T.G. Gesner

Erythropoietin (EPO) biosynthetically labelled with $[^{35}S]$cysteine was produced from Chinese hamster ovary (CHO) cells containing amplified copies of human EPO cDNA. The glycosylated recombinant $[^{35}S]$EPO, purified to virtual radiochemical homogeneity, was biologically active. We studied the interaction of this labeled recombinant EPO with erythroid precursor cells from mice made anemic with phenylhydrazine. The $[^{35}S]$-labeled molecule bound to erythroid precursors in a time- and temperature-dependent manner. The binding was specific for EPO, and neither insulin, transferrin, epidermal growth factor, nor multiplication stimulating activity could compete for EPO binding sites. In the presence of 0.2% sodium azide, which blocks 80% to 90% of internalization, the recombinant molecule bound with an apparent K_d of 750 pmol/L and 100 to 200 binding sites per cell at 37°C. Asialo-EPO was a more effective competitor than sialated EPO for the available binding sites. Thus, the enhanced biological specific activity of asialo-EPO could result from its enhanced binding affinity. We also studied recombinant human EPO labeled with ^{125}I and found that it also bound to the erythroid cells in a saturable and specific manner. After 90 minutes of incubation at 37°C, most of the bound $[^{35}S]$EPO was internalized, whereas most of the $[^{125}I]$EPO remained on the cell surface. The reduced internalization of the iodinated molecule could account for the previously reported functional deficit associated with iodination.

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THE MAJOR regulator of mammalian erythropoiesis is a glycoprotein called erythropoietin (EPO). This hormone is believed to be synthesized by cells in the kidney, where its production is controlled by the blood oxygen tension.[1] The erythropoietin released from the kidney interacts with erythroid precursor cells to stimulate their survival, growth, and differentiation. Investigation of the mechanism of action of EPO has been hindered both by the limited availability of the pure hormone and by the fact that iodination causes the loss of biological activity.[2] The lack of radiolabeled EPO that is biologically active has especially hindered studies of the interaction of erythropoietin with its target cells. Recently, however, specific and saturable high-affinity EPO binding sites on Friend virus-transformed mouse erythroblasts were detected using EPO labeled by carbohydrate tritiation.[3]

We have reported the cloning of a full-length human EPO cDNA and expression of the biologically active recombinant hormone from mammalian cells.[4] The recombinant molecule has the same in vivo and in vitro properties as the naturally occurring protein purified from the urine of patients with aplastic anemia.[4] The availability of genetically engineered mammalian cells producing high levels of fully glycosylated recombinant EPO allows production of biologically active radiolabeled EPO by cellular biosynthesis using, for example, $[^{35}S]$labeled amino acids. In this article, we report the use of $[^{35}S]$cysteine-labeled recombinant human EPO to investigate the binding of EPO to anemic mouse erythroid precursors accumulated during phenylhydrazine-induced anemia. Previous work by Krantz and Goldwasser was restricted to extrinsically labeled protein and to virally transformed cells.[3] Our data demonstrate that EPO binds to saturable high-affinity sites and moves from the cell membrane into the cell interior. Finally, we compare the interaction of $[^{125}I]$-labeled recombinant EPO with cells to that of $[^{35}S]$ internally labeled EPO and suggest that iodination may specifically affect the internalization step.

MATERIALS AND METHODS

Preparation of erythroid precursor cells. F_1 generation female mice from a C57BL x C3H cross (Jackson Labs, Bar Harbor, ME), (aged 6 to 8 weeks) were made anemic by phenylhydrazine injections.[5] Three to 5 days after the last injection, the animals were killed by cervical dislocation, and the enlarged spleens were aseptically removed and teased apart in nucleoside-free α-medium supplemented with penicillin and streptomycin, (α + P/S) (GIBCO). The resulting crude suspension was passed through 18-g and 21-g needles (Becton Dickinson) to produce a uniform single-cell suspension. The few remaining clumps were permitted to settle out. The supernatant was centrifuged at 600 g for 5 minutes. The pellet was suspended in 2 mL of NH_4Cl-Tris buffer to lyse mature erythroid cells osmotically, diluted to 15 mL with α + P/S, and recentrifuged for 5 minutes at 600 g. The cells were finally suspended in α + P/S + 20% fetal bovine serum, 0.1 mmol/L of β-mercaptoethanol (binding medium) at a density of 1 to 4 × 10^8 cells/mL. The resulting cells are 90% erythroid precursors.[5]

Specific binding assays. The assay for specific binding of radiolabeled EPO to erythroid precursors was essentially that of Krantz and Goldwasser.[3] In brief, EPO (200 to 500 pmol/L) was added in binding medium to a 96-well U-bottom tissue culture plate (Costar, Cambridge, MA). Cells were added such that the final volume per assay was 100 μL. Incubations were performed for 90 minutes at 37°C in a 5% CO_2 atmosphere unless otherwise noted. Ice-cold phosphate-buffered saline (PBS) (100 μL) was then added, and the entire plate was transferred to an ice-water bath. The contents of each well were layered over 350 μL of fetal bovine serum (FBS) and centrifuged in a Fisher microfuge for 1 minute. After quick freezing at −70°C, the tips of the microfuge tubes were cut just above the pellets. Pellets were solubilized in 0.1% sodium dodecyl sulfate (SDS) (500 μL) and counted for 15 minutes in 10 mL Aquasol-2 (New England Nuclear). Assays for nonspecific binding were run in which the assay mixture contained a 100-fold excess of unlabeled EPO. The radioactivity bound in the assays with excess unlabeled EPO was considered nonspecific and was subtracted from the total binding to yield the specific binding. Assays for both total and

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nonspecific binding were always conducted in duplicate. The range for replicates for each datum point used was ≤10% of the mean.

Epidermal growth factor, insulin, transferrin, and multiplication stimulating activity were obtained commercially from Sigma Chemicals (St Louis).

Assay for EPO internalization. To discriminate surface-bound EPO from internalized EPO, the widely used low pH/salt method was used.[6-8] In brief, the cells centrifuged through the FBS cushion in the binding assay were resuspended in 0.05 mol/L of glycine-HCl buffer pH 3.0 containing 0.15 mmol/L of NaCl and were incubated at 0°C for 5 minutes. The cells were then centrifuged, and the solubilized pellets and supernatants were counted. Inhibitors of oxidative phosphorylation are as effective as incubation at 0°C in inhibiting receptor ligand internalization. In cells in which internalization had been blocked with sodium azide, >90% of radioactivity was released by washing cells with low pH/salt buffer. All the released radioactivity was TCA precipitable; thus, the low pH released undergraded EPO. Washing azide-treated cells with pH 7.4 did not release any radioactivity. These data are consistent with experiments on release of other ligands.[6-8] The supernatant radioactivity therefore represents surface-bound ligand, and cell-associated radioactivity represents internalized ligand. In experiments in which [^{35}S]-labeled EPO was used, cell pellets were solubilized with 0.1% SDS; in experiments in which [^{125}I]EPO was used, cell pellets were counted directly.

Biosynthetic labeling with ^{35}S. A line of Chinese hamster ovary cells (CHO) containing highly amplified copies of the human EPO cDNA and selected for high levels of functional EPO expression was used. Development of such CHO cells and the structure of the expression vectors used has been described previously.[9,10] The cells were grown attached to T-125 cell-culture flasks until they were 90% confluent. They were then rinsed with PBS and placed in cysteine-deficient Dulbecco's medium containing 0.5 mCi/mL of [^{35}S]cysteine (New England Nuclear, Boston). The cells were permitted to synthesize and secrete [^{35}S]EPO for a 6-hour period. The conditioned medium was collected, and the [^{35}S]EPO was purified from this medium.[11] The purified EPO was >95% radiochemically pure as determined by polyacrylamide gel electrophoresis (PAGE).

The mass of [^{35}S]-labeled hormone was determined by radioimmunoassay (RIA) using a nonradioactive recombinant EPO standard, whose mass was determined by amino acid analysis, and rabbit polyclonal antibody made against purified CHO-expressed EPO. The radiospecific activity was comparable to that determined by the self-displacement assay.[12] The [^{35}S]-labeled hormone had a specific biological activity of 250,000 U/mg of protein as determined in the Krystal bioassay[5] (further details of biological specific activity are given in the Discussion section).

Iodination of EPO. [^{125}I]-Labeled EPO was prepared by the chloramine T method.[13] EPO (700 ng) was iodinated in 0.5 mol/L of sodium phosphate pH 7.4 with 1 mCi of [^{125}I] and 40 μg of chloramine T (final reaction volume is 125 μL). After a 1-minute incubation at room temperature, 240 μg of sodium metabisulfite and 1 mg of KI was successively added (final mixture volume 475 μL). The reaction mixture was then immediately applied to a column of PD10 resin (Pharmacia), and the protein peak from this column was applied to a Sephadex G-100 column. The EPO peak from this column was collected.

Preparation of asialo-EPO. Purified recombinant human EPO was acid-desialated using conditions previously described by Goldwasser and colleagues.[14] In brief, the hormone was heated at 80°C for 30 minutes with 30 mmol/L of HCl to remove essentially all the sialic acid.

Biological activity of ^{35}S and [^{125}I]EPO. [^{35}S]EPO (50 mU) and [^{125}I]EPO were assayed in the Krystal cell proliferation assay.[5] Assay results revealed that the [^{35}S]EPO had the expected biological activity but that the [^{125}I]EPO had <10% of the expected activity.

RESULTS

Careful determination of the specific activity of the [^{35}S]-labeled EPO was necessary to measure the binding characteristics of our recombinant EPO to erythroid precursor cells accurately. A self-displacement analysis[12] comparing the binding of increasing amounts of labeled EPO and the competition of a fixed amount of labeled EPO with increasing concentrations of unlabeled EPO is shown in Fig 1. The analysis yielded a specific activity of 150 to 250 cpm/fmol. This specific activity was sufficient to allow binding studies using ~10^7 spleen erythroid cells per assay.

Figure 2 shows the binding of EPO to erythroid precursor cells at 37°C. Specific binding reached a plateau by 60 minutes, whereas nonspecific binding continued to rise in a linear fashion during the entire course of the experiment. At 10°C binding was much slower and never reached the levels obtained at 37°C (data not shown). Experiments performed at 0°C showed that EPO binding was further reduced; in addition, over several hours cell clumping was observed. The specific binding at 0°C was so low and the cell clumping so severe that it was not possible to conduct quantitative binding studies at this reduced temperature. Table 1 shows that of a variety of polypeptide growth factors tested only EPO could quantitatively compete for binding to normal erythroid precursors. As shown in Table 1, specific binding usually represented 60% of the total binding.

Because it was necessary to do binding experiments at 37°C to obtain sufficient amounts of specifically bound

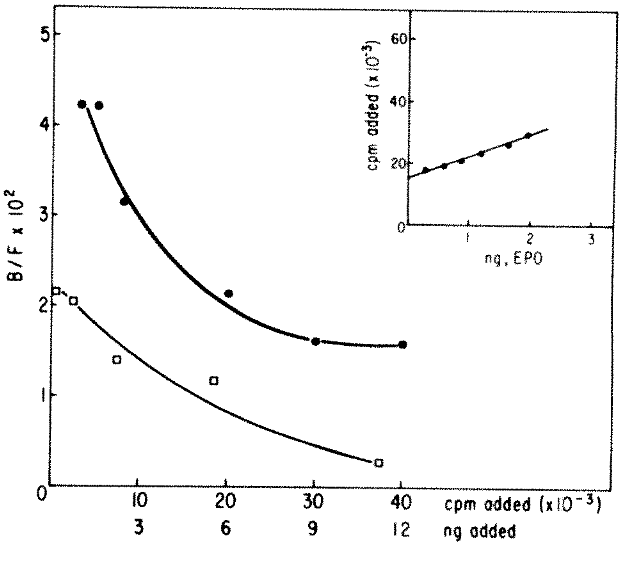

Fig 1. Self-displacement analysis of [^{35}S]-labeled erythropoietin (EPO). Increasing amounts of [^{35}S]EPO were added to anemic mouse spleen cells in one assay set (□). In the other assay set, increasing amounts of amino acid-analyzed Chinese hamster ovary (CHO)-expressed EPO were added to 2 × 10^3 cpm of [^{35}S]-labeled EPO (●). Each assay set contained 1.6 × 10^7 cells. Inset shows total radioactivity added to the binding system plotted against the corresponding mass of EPO, using several points at which the ratios of bound to free were the same.

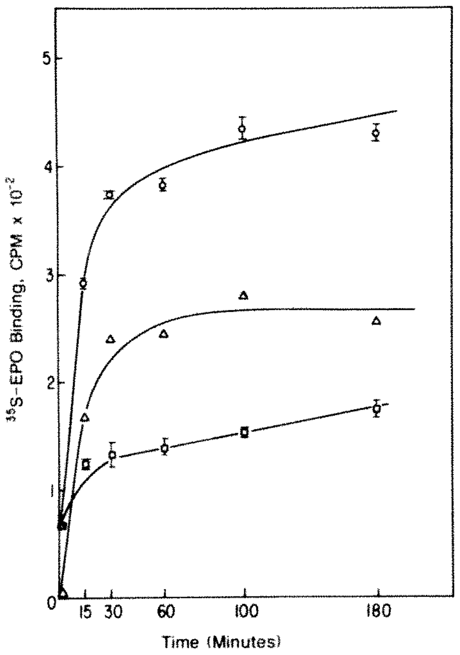

Fig 2. Time-course of specific binding of [^{35}S]erythropoietin (EPO) to mouse spleen erythroid precursors. [^{35}S]-labeled EPO (167 pmol/L) was incubated with 2.2 × 10^7 anemic mouse spleen cells in cell culture medium and incubated at 37°C. At the indicated time, specific binding of EPO was measured as described in the Materials and Methods section. Total binding (O); nonspecific binding (□); specific binding (Δ). Each point is the mean of duplicate determinations. Similar results were obtained in two different experiments. Vertical bars represent range of values for each mean.

[^{35}S]EPO molecules to analyze binding isotherms accurately, experiments were performed with 0.2% azide. Inhibitors of oxidative phosphorylation block receptor internalization as well as incubation at 0°C.[6] Table 2 shows that this concentration of sodium azide blocked internalization of [^{35}S]EPO by 80% to 90%. Figure 3 shows the binding isotherm at 37°C with 0.2% azide and a Scatchard transformation of the data.[15] Because the small amount of residual internalization could affect the binding equilibrium, we emphasize that our binding constants should be considered apparent. In two separate experiments performed in duplicate, the apparent mean K_d was 750 pmol and the number of binding sites per cell was 100 to 200. The difference between the K_d and the concentration required for half-maximal biological activity could indicate that only a very small amount of receptors must be occupied to achieve activation of the cells.

Table 1. Growth Factor Competition for EPO Binding Sites

Competitor	[^{35}S]EPO cpm Bound
None	849
EPO	331
EGF	879
Insulin	866
Transferrin	824
Multiplication - Stimulating activity	814

Binding assays were performed as described in the Materials and Methods section. Assays were performed using either [^{35}S]-labeled erythropoietin (EPO) or [^{35}S]-labeled EPO plus a 100-fold excess of cold growth factor. Results are presented as total counts per minute of [^{35}S]EPO bound. The experiment was repeated twice with similar results.

Table 2. Effect of Sodium Azide on EPO Internalization

	Internalized Specifically Bound [^{35}S]EPO (cpm Bound)	
Addition	Exp. 1	Exp. 2
Control	658	481
Sodium azide (0.2%)	55	110

Mouse erythroid precursor cells were preincubated either with or without sodium azide for 60 minutes at 37°C. Cells were then incubated with [^{35}S]erythropoietin (EPO) for 90 minutes at 37°C. Assays were performed using either labeled EPO or labeled EPO plus a 100-fold excess of unlabeled EPO to determine specific binding. At the end of the incubation, cells were pelleted through a fetal bovine serum cushion and washed in pH 3.0/0.15 mol/L of sodium acetate buffer. Low pH washed cells were then centrifuged and counted for radioactivity.

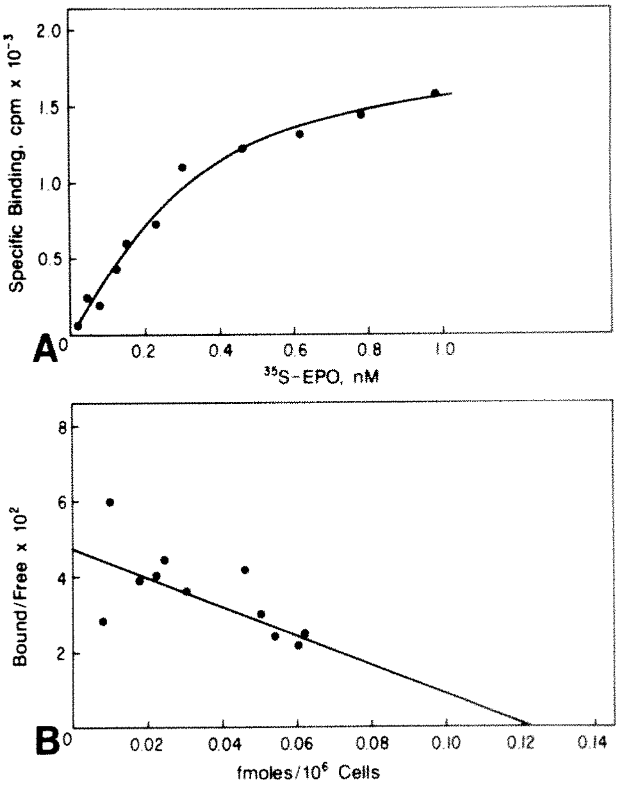

Fig 3. (A) Effect of increasing [^{35}S]erythropoietin (EPO) concentration on specific binding to anemic mouse spleen cells. Increasing concentrations of [^{35}S]EPO were incubated with 10^7 anemic mouse spleen cells at 37°C for 90 minutes with 0.2% sodium azide. At the end of the incubation period, specific binding was determined as described in the Materials and Methods section. (B) A Scatchard transformation of the data is also shown. Each point is the mean of duplicate determinations. The experiment was repeated twice with similar results.

Goldwasser and colleagues[14] reported that asialo-EPO has a greater in vitro activity than the fully sialated molecule. We used acid-desialated recombinant human EPO to confirm the increased biological activity of asialo-EPO. The mass of sialo and asialo-EPO added was determined by RIA. In preliminary experiments, 0.5 μg of EPO was subjected to acid desialation (30 mmol/L of HCl, 80°C, 30 minutes) and another 0.5 μg was heated to 80°C for 10 minutes as a control. The RIA measured 0.5 μg/mL for both the control sialated material and the acid-desialated material. The sialo-EPO was more effective in stimulating cell proliferation on a molar basis in the spleen cell proliferation assays (Fig 4). It was therefore of interest to compare the binding of sialo- and asialo-EPO with erythroid precursor cells. Figure 5 shows that with increasing concentrations of unlabeled competitor, the asialo-molecule was a more effective competitor for EPO binding sites than the fully sialated EPO. Thus, an increased affinity of the asialo-molecule for the EPO receptor could account for its increased in vitro biological activity.

Although the [^{125}I]EPO does not possess biological activity,[2] there are no reports of [^{125}I]EPO binding activity toward erythroid precursor cells. We therefore investigated the binding of ^{125}I recombinant EPO that had been iodinated with the chloramine T method. Goldwasser showed that the chloramine T reaction itself does not alter the biological activity of EPO, but the actual addition of the bulky ^{125}I atom ablates the biological activity.[2] This material, however, still cross-reacts with antibodies to native EPO.[2] The specific activity of the iodinated molecule used in these experiments was 3,000 to 4,000 cpm/fmol, as determined by self-displacement analysis. Figure 6 shows that the [^{125}I]-labeled EPO binds to anemic mouse spleen cell precursors in a time-dependent manner and the binding is specific. Binding

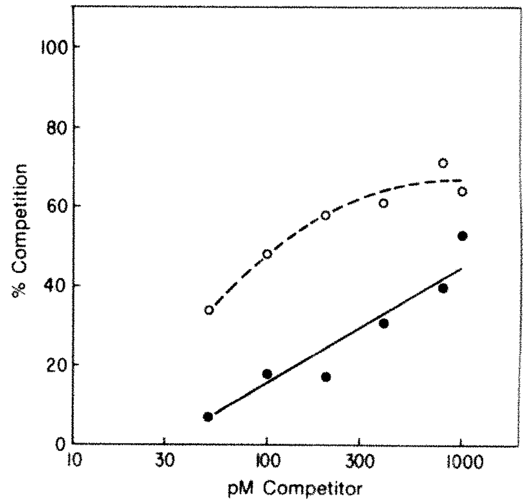

Fig 5. Comparison of sialated and asialo-erythropoietin (EPO) for competition at EPO binding sites. Sialated and asialo-EPO were prepared as described in the Materials and Methods section. Incubation mixtures contained 3.1 × 10^7 cells, increasing concentrations (3 to 1,000 pmol/L) of the EPO preparations, and 140 pmol/L [^{35}S]-labeled EPO. Cells were incubated for 90 minutes and, at the end of incubation, specific binding was determined. At each concentration of competitor, percentage of competition was calculated using the following equation: percentage of competition = total binding minus counts bound in the presence of a given concentration of competitor divided by total binding and multiplied by 100. Competition with sialated EPO (●); competition with asialo-EPO (○). The experiment was performed twice with similar results.

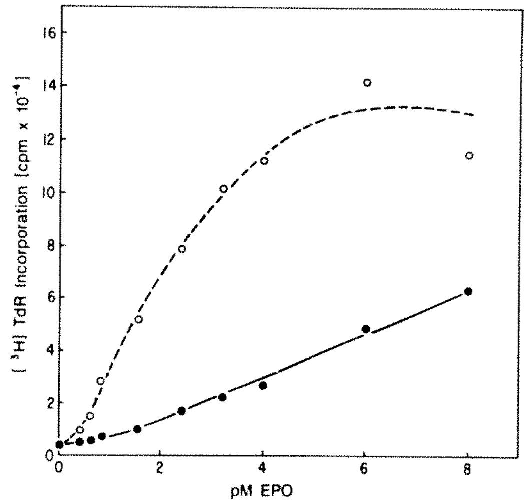

Fig 4. Bioassay of sialated *v* asialo-erythropoietin (EPO). Increasing concentrations of either sialated (●) or asialo (○) recombinant EPO were added to the Krystal bioassay system[5] using anemic mouse spleen cells. Following 18 hours of exposure to EPO, cells were pulsed with [^{3}H]thymidine, and incorporation of radioactivity into DNA was determined. Assays were performed in triplicate, and the experiment was performed twice with similar results.

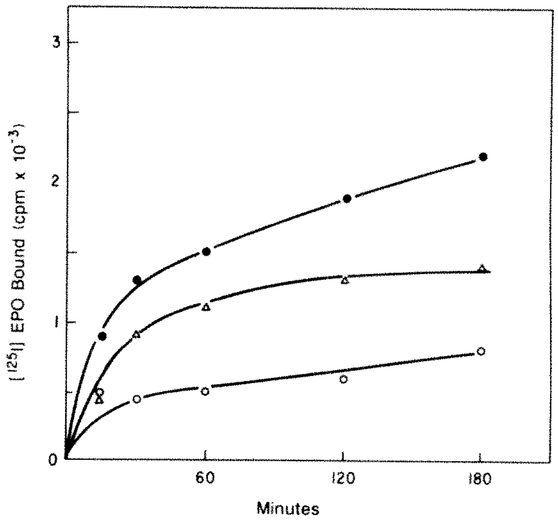

Fig 6. Specific binding of iodinated erythropoietin (EPO) to anemic mouse spleen erythroid precursor cells. [^{125}I]EPO (460 pmol/L) was incubated with 10^7 spleen cells for increasing amounts of time at 37°C. At the indicated times, cells were harvested and specific binding was determined as described in the Materials and Methods section. Total binding (●); specific binding (Δ); nonspecific binding (○). The experiment was performed twice with similar results.

Table 3. Subcellular Distribution of [^{35}S]- and [^{125}I]-Labeled EPO Bound to Mouse Erythroid Precursor Cells

Labeled EPO	Ratio of Surface to Internalized Specifically Bound EPO Exp. 1	 Exp. 2	 Exp. 3
	S/I	S/I	S/I
[^{125}I]EPO	4.0	4.0	2.8
[^{35}S]EPO	0.43	0.35	0.59

Mouse erythroid precursor cells were incubated for 90 minutes at 37°C with either [^{125}I]erythropoietin (EPO) or [^{35}S]EPO. Assays were performed using either labeled EPO or labeled EPO plus a 100-fold excess of unlabeled EPO to determine specific binding. Cells were then pelleted through a fetal bovine serum cushion and washed in pH3.0/0.15 mol/L of sodium acetate buffer. Cells were again centrifuged, and the wash supernatants and all pellets were counted for radioactivity. Counts in the wash supernatant are from the cell surface (S) and those in the pellet are internalized (I).

experiments were also conducted using increasing concentrations of iodinated EPO. Scatchard transformations of binding isotherms from two different experiments (data not shown) allowed calculation of an apparent dissociation constant of 1.7 nmol/L and 165 binding sites per cell.

A number of polypeptide hormones are internalized after they interact with their receptors.[6-8] We were therefore interested in the subcellular distribution of labeled EPO after its binding to erythroid precursors. We compared the distribution of radioactivity (surface *v* internalized) on the cells 90 minutes after binding at 37°C using the low pH salt wash method.[6-8] Internalization studies comparing the distribution of [^{125}I]EPO with [^{35}S]EPO showed that the ratio of surface-bound/internalized EPO differed by tenfold between [^{35}S]- and [^{125}I]-labeled hormone (Table 3). The [^{35}S]EPO appeared to be mainly internalized, whereas the iodinated hormone was found almost exclusively on the cell surface. Three different experiments produced the same result. Therefore, the iodinated molecule appears to bind to erythroid progenitor cells, but the iodine moiety appears to interfere with the internalization phenomenon.

DISCUSSION

These studies showed that normal erythroid precursor cells have high-affinity saturable binding sites for EPO. The number of binding sites and the affinity constants are lower than that observed in the virus-transformed erythroid precursors using human urinary EPO, possibly because Krantz and Goldwasser[3] calculated specific activity from counts per minute per unit of biological activity, assuming 70,000 U/mg of protein for purified human urinary EPO. Our analysis indicates that purified recombinant EPO has an in vitro specific biological activity of 250,000 U/mg of protein. This difference in specific biological activity could account for the difference in binding affinity and receptor number between our data and previously published results.[3] The viral transformation of the cells used by Krantz and Goldwasser[3] could also contribute to the difference in number of binding sites. The small number of EPO receptors on erythroid precursor cells is similar to that for G–colony-stimulating factor (CSF) binding sites on granulocytes from marrow (50 to 500 sites/cell),[16] and CSF-2α binding sites on a bone marrow cell line (400 sites/cell).[17] Thus, low receptor number may be characteristic of hematopoietic growth factor receptors.

The availability of a binding assay using intrinsically labeled recombinant EPO has allowed examination of the effect of sialic acid residues on the interaction of EPO with its target cells. Goldwasser and colleagues[14] and Schooley[18] reported that removing the sialic acid residues from EPO causes a significant increase in its activity in vitro. We confirmed that recombinant EPO that has been desialated also has greater biological activity, on a molar basis, than does sialated recombinant EPO. In addition, we showed that asialo-EPO is a more effective competitor for EPO binding sites than is the sialated molecule. These studies were also confirmed using biosynthetically produced asialo-EPO expressed in a CHO cell variant that does not sialate glycoproteins (unpublished observations). Thus, the asialo-EPO probably has a higher affinity for the EPO receptor than does the fully sialated molecule. If the target cell membrane is negatively charged, a molecule carrying a cluster of anionic groups may be inhibited in its ability to interact with the cells. The asialo-hormone would have easier access to the receptor. These data are consistent with the behavior of desialated human gonadotrophins. Thus, desialated human luteinizing hormone and chorionic gonadotrophin show increased binding affinity for rat testes membranes and enhanced in vitro biological activity.[19]

[^{125}I]-Labeled EPO bound to erythroid cells with characteristics similar to those of the [^{35}S]-labeled molecule. The iodinated EPO, however, was only poorly internalized as compared with the [^{35}S]-labeled molecule, and most of the bound factor remained on the cell surface. The poor internalization of the [^{125}I]-labeled molecule may contribute to its lack of biological activity; however, other possibilities cannot be excluded.[20] Further comparisons of [^{125}I]-labeled EPO to the intrinsically labeled molecule might contribute to an understanding of the mechanism of action of EPO.

These results demonstrate that recombinant human EPO interacts with erythroid precursor cells in terms of binding and biological activity as well as natural human EPO. It is therefore reasonable to begin to consider the possible clinical uses of purified recombinant EPO. These data should be helpful in developing recombinant human erythropoietin for clinical use in appropriate anemias.

ACKNOWLEDGMENT

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Human Monoclonal Antibody Against Rh(D) Antigen: Partial Characterization of the Rh(D) Polypeptide From Human Erythrocytes

By Christian Bloy, Dominique Blanchard, Patrick Lambin, Dominique Goossens, Philippe Rouger, Charles Salmon, and Jean-Pierre Cartron

A human monoclonal anti-Rh(D) antibody produced by an Epstein-Barr virus (EBV)-transformed B-cell line (IgG$_1$(λ), clone H2D5D2) has been purified on protein A-Sepharose column and used for binding studies and immune precipitation of the blood group rhesus (Rh) antigens. Scatchard plot analyses show that the ^{125}I-labeled antibody (iodo-gen procedure), binds to 1.09 × 10^5, 0.43 × 10^5, and 0.32 × 10^5 antigen sites on each D--/D--, R^{2}R^2 and R^{1}R^1 RBC, respectively, with an association constant of ~0.6 × 10^8 mol/L^{-1}. Immune precipitation studies indicate also that the Rh(D) antigen of the Rh(D)-positive RBCs is carried by a 29 kd polypeptide as deduced from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). No material could be precipitated from Rh(D)-negative or Rh$_{null}$ RBCs. These results indicate that the monoclonal and the polyclonal human anti-Rh(D) behave similarly. A sample (Blo., presumed genotype R^{2}r or R^{0}R^2) showing an increased number of antigen sites (0.76 × 10^5/cell) and a high binding constant (5.7 × 10^8 mol/L^{-1}) was used, as well as D--/D-- RBCs, for further purification of the 29-kd component. Extraction by Triton X-100 (0.1% to 5%) of the immune complexes formed between the membrane-bound Rh(D) antigens and the monoclonal antibody as well as a direct quantitative estimate of the 29-kd component, suggest that the Rh(D) polypeptide is loosely bound to the skeleton, since ≤80% can be solubilized from the membrane. In similar conditions, glycophorin A showed a slight association with the Triton-insoluble residue, whereas glycophorin B was easily and completely extracted. In contrast, both the minor RBC sialoglycoproteins, glycophorin C and glycoprotein γ, remained predominantly bound to the membrane skeleton. The purified Rh(D) polypeptide obtained from Blo. and D--/D-- RBCs by immunoprecipitation and preparative gel electrophoresis was homogenous as judged by SDS-PAGE. Amino acid composition indicated that the Rh(D) protein contained sulfhydryl groups which are essential for biological activity.

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ELUCIDATION of the chemical nature of the blood group Rhesus (Rh) antigens is of primary interest since these antigens are highly immunogenic in humans and are frequently involved as targets of antibodies produced in hemolytic disease of the newborn and autoimmune hemolytic anemia.[1-3] Until now, however, attempts to purify the Rh antigens have been unsuccessful, presumably because they are restricted to higher primate and human erythrocytes and are present in relatively small number on the RBC membrane.[4,5] In addition, considerable evidence shows that the Rh antigens belong to cysteine-containing polypeptides requiring lipid association for full activity[6-10] and that the Rh antigenic expression can be modulated by altering the cholesterol-phospholipid content of erythrocyte membranes.[11,12]

Recently, the Rh(D) antigen was shown to be associated with an integral membrane protein of 28- to 33-kd apparent mol wt[13,14] presumably devoid of carbohydrates[15] but tightly bound to the RBC membrane skeleton.[16,17] Interestingly, some rare individuals (Rh$_{null}$ and Rh$_{mod}$) whose RBCs are characterized by the absence of, or a severe reduction of all the Rh antigens (D, C, c, E, e, and others)[18,19] have a mild hemolytic anemia, stomatocytosis, and reticulocytosis.[20,22] This suggests that Rh-deficient RBCs exhibit a membrane defect[23] and that Rh antigens might represent the first example of a blood group structure needed for preserving normal RBC function.

Obviously the development of monoclonal antibodies directed against Rh structures will provide new tools to study these antigens, as well as potential reagents for diagnostic purposes and prophylaxis of Rh hemolytic disease. To date, only a few examples of human monoclonal antibodies to Rh(D) antigen have been reported.[24-27] We recently produced such an antibody from a highly stable Epstein-Barr virus (EBV)-transformed human B cell line,[27a] and this reagent was used for study of Rh antigens and purification of the Rh(D) polypeptide from human RBCs.

MATERIALS AND METHODS

Materials. Reagent grade chemicals were used throughout. Protein A-Sepharose CL 4B (2 mg/mL of gel) was obtained from Pharmacia (Uppsala, Sweden) and *Staphylococcus aureus* absorbent was obtained from Miles-Yeda (Naperville). Iodo-gen (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril) was bought from Pierce (Rockford, IL). ^{125}INa (625 MBq/μg of iodine) was from the Radiochemical Centre (Amersham, Bucks, England). Triton X-100 and bovine serum albumin (BSA) were purchased from Serva (Heidelberg, FRG) and from Sigma Chemicals (St Louis), respectively. Dibutylphthalate and *bis*-2-ethyl-hexylphthalate were obtained from Fluka (Buschs, Switzerland). Red cells from donors of known Rh genotype (rr, R^{1}R^1, R^{2}R^2, R^{2}r″) and from donor Blo. (probable genotype R^{2}r or R^{2}R^0) were collected at the Centre National de Transfusion Sanguine (Paris, France). Rare RBCs (D--/D--, Gou.; Rh$_{null}$ amorphic type, Fri.) were obtained from the Centre National de Référence sur les Groupes Sanguins (Paris) and were stored in liquid nitrogen until used.

Purification of the human anti-Rh(D) monoclonal antibody. The human monoclonal anti-Rh(D) antibody [IgG$_1$(λ)] was produced by an EBV-transformed cell line (H$_2$D$_5$D$_2$ clone) obtained from the B lymphocytes of an immunized donor.[27a] The monoclonal antibody was purified from the cell culture supernatant by protein A-Sepharose chromatography essentially as described.[28] The tenfold concentrated supernatant (YM10 membrane, Amicon, Danvers,

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MA) was equilibrated in 100 mmol/L of sodium phosphate, 150 mmol/L of NaCl, pH 8.0, and was applied to a 10 mL of protein A-Sepharose column at room temperature and sequentially eluted at pH 6.5 (100 mmol/L of sodium phosphate, NaCl 150 mmol/L) and at pH 4.5 (100 mmol/L of sodium acetate buffer). The IgG_1 molecules were finally eluted with 100 mmol/L of glycine, pH 2.8, and quickly neutralized using a 100 mmol/L Tris solution. Biological activity of the preparation was checked by a direct agglutination test performed with 50 μL of a 3% (vol/vol) papain-treated RBC suspension and 50 μL of the immunoglobulin fractions serially diluted in 150 mmol/L of NaCl. Purity of the material obtained was controlled by PAGE in nonreducing conditions[29] and gel permeation on a TSK G3000 SW column (LKB, Bromma, Sweden) developed at 100 μL/mn in Tris acetate 20 mmol/L, pH 7.2, 150 mmol/L of NaCl.

Binding of labeled anti-Rh(D) to human erythrocytes. The purified monoclonal anti-Rh(D) (500 μL, 1.5 mg/mL) was ^{125}I-labeled by the iodo-gen procedure as described below, with 7.4 MBq ^{125}INa at room temperature. Free iodine was eliminated by gel filtration on a Sephadex G-25 column (15 × 1.5 cm) equilibrated in phosphate-buffered saline (PBS) (10 mmol/L of phosphate buffer, pH 7.2, 150 mmol/L of NaCl). The IgG fraction eluted in the void volume was used for the binding experiments. More than 95% of the material was trichloroacetic precipitable with a specific activity of 7.4 kBq/μg of protein. Binding studies to RBCs were carried out in 0.4-mL polypropylene microcentrifuge tubes essentially as described by Briles and colleagues.[30] Fifty microliters of PBS containing 1.5×10^6 cells was incubated, in duplicate, with 0.017 to 6 μg of ^{125}I-labeled anti-Rh(D) and 2% (wt/vol) BSA for 1 hour at room temperature. The RBCs were then washed once in PBS containing 2% (wt/vol) BSA and further separated from unbound anti-Rh(D) molecules by the addition of 100 μL of oil (dibutylphthalate/*bis*-2-ethyl-hexylphthalate, 3/1, by vol) followed by centrifugation of the tubes. Bound radioactivity was determined by counting the pellet in a LKB γ counter (model 1274). Estimation of the number of antigen sites of each cell and the association constant for the anti-Rh(D) was calculated according to Scatchard.[31]

Immune precipitation and purification of the Rh(D) protein. Immune precipitation of Rh (D) antigen from ^{125}I-labeled Rh-positive RBC membranes was performed essentially according to Gahmberg.[15] Fifty microliters of packed cells were suspended in 1 mL of PBS and labeled with 9.25 MBq ^{125}INa using iodo-gen coated on plastic flasks (100 μg per flask) as oxidizer.[32] The labeled cells were washed in PBS and mixed with 50 μL of unlabeled carrier packed cells. Membranes were prepared according to Steck and Kant,[33] washed once with PBS (this treatment removed protein band 6), and incubated for 1 hour at 4°C with 20 μL (7 to 14 μg) of the purified IgG fraction from the monoclonal anti-Rh(D) antibody. The coated membranes were then washed twice with PBS and solubilized following gentle agitation for 30 minutes at 4°C with 300 μL of 5% (wt/vol) Triton X-100 in PBS. Following centrifugation at 37,000 g (30 minutes), the supernatant containing the immune complexes were incubated for 1 hour at +4°C with 100 μL of a 10% (wt/vol) *Staphylococcus aureus* suspension in PBS containing 0.5% (wt/vol)Triton X-100. Pellets obtained by centrifugation in the cold (5 minutes at 10,000 rpm) were carefully suspended in 200 μL of 0.5% (wt/vol) Triton X-100 in PBS and applied to a sucrose gradient composed of 400 μL of 10% (wt/vol) sucrose in PBS containing 0.5% (wt/vol) Triton X-100 layered on 800 μL of 20% (wt/vol) sucrose in PBS. The pellet was resuspended in 200 μL of PBS-Triton and washed several times on the sucrose gradient. Specifically bound proteins were eluted from the final pellet by boiling in 100 μL of 10 mmol/L of Tris-HCl, 1 mmol/L of EDTA, pH 6.8, containing 5% (wt/vol) SDS. The eluted material was analyzed by SDS-PAGE performed in a 10% (wt/vol) polyacrylamide gel in a discontinuous buffer system[34] and identified by autoradiography on Cronex 4 film with intensifying screen (Dupont de Nemours, Wilmington, DE). Preparative purification of the Rh(D) polypeptide was made by repetition of the same protocol except that protein A-Sepharose CL 4B (100 μL of settled gel in Eppendorf tube) was used instead of the *S aureus* absorbent. The protein eluted from the absorbent was further purified on a preparative SDS-PAGE in a 10% (wt/vol) gel (3 mm thick) which was pretreated according to Wiener and colleagues.[35] To reduce contamination by glycine in the preparation, a Tris-borate buffer (50 mmol/L of Tris, 60 mmol/L of boric acid, pH 8.3, containing 0.1% SDS) was used during gel electrophoresis.[36] Electroelution of the Rh(D) protein from the pieces of gel inserted in a dialysis bag was done at 4°C in 30 mmol/L of Tris acetate buffer, pH 7.4, containing 0.1% SDS (150 mA, for 8 hours) using a homemade electrophoretic cell and two platinum electrodes connected to a power supply (Apelex, France). The purified material was analyzed by SDS-PAGE in nonreducing conditions, and quantitated by R 250 Coomassie blue staining,[37] taking BSA as standard protein.

Solubilization of the Rh(D) protein with a nonionic detergent. Red cells coated with ^{125}I-labeled anti-Rh(D) (1.5×10^6 R^2R^2 cells incubated with 1 μg antibody) were washed four times in PBS-BSA and mixed with 50 μL of unlabeled RBCs. Membranes were prepared by hypotonic lysis in a 5 mmol/L of phosphate buffer, pH 8.0,[33] washed once in PBS, and the immune complexes were solubilized following addition of 3 vols of 0.1% to 5% (wt/vol) Triton X-100 in PBS and gentle shaking (10 rpm) at 4°C for 30 minutes. The distribution of the ^{125}I-labeled antibody was determined by counting the supernatants and the pellets obtained after centrifugation at 37,000 g for 30 minutes at 4°C. In addition, the distribution of the Rh(D) polypeptide between the Triton X-100 soluble and insoluble fractions was also investigated. Membranes obtained from ^{125}I-labeled erythrocytes (Blo.) were washed once in PBS and solubilized with variable amounts of Triton X-100 (0.1% to 5%, wt/vol) as described above. Supernatants and pellets recovered by centrifugation (37,000 g for 30 minutes at 4°C) were analyzed by SDS-PAGE as above. The radioactivity incorporated in the Rh(D) polypeptide was determined by cutting out the labeled band localized by autoradiography of the dried gel. For comparison, the distribution of glycophorins A, B, C, and glycoprotein γ was estimated after periodic-acid Schiff (PAS) staining of the gels.[37]

Amino-acid analysis. Samples of purified Rh(D) polypeptide were concentrated and desalted by extensive dialysis on a Centricon 10 system (Amicon, Danvers, MA). Aliquots containing 10 μg of purified protein were hydrolyzed in 6N HCl (with 0.2% phenol) at 110°C in sealed evacuated tubes for 18 hours, and the hydrolysates were analyzed with a Biotronik LC 5001 amino acid analyzer. Methionine and half-cysteine were determined in a separate aliquot as methionine sulfone and cysteic acid after performic oxidation.

RESULTS

Purification of the monoclonal antibody and binding studies. About 3 mg of biologically active monoclonal antibody was recovered in the acid eluate of the protein A-sepharose column from 300 mL of cell culture supernatant.[1] The purified IgG antibody, which gave a single band on SDS-PAGE and following gel filtration on TSK G3000 SW column (not shown), strongly agglutinated papain-treated Rh-positive (R^2R^2) RBCs at 0.3 nmol/L concentration, whereas there was no agglutination of papain-treated Rh-negative cells (rr) even at a concentration of 40 nmol/L (optimum pH was 7.0 to 8.0). Absorption experiments on Rh(D)-positive and Rh(D)-negative RBCs indicated that >90% of the IgG molecules from the purified preparation were associated with anti-Rh(D) activity.

After ^{125}I-labelings the monoclonal antibody retained its biological activity, and the binding to native Rh-positive RBCs occurred without agglutination and was saturable (Fig 1, inset). The binding data plotted according to Scatchard[31] indicated that R^1R^1 and R^2R^2 erythrocytes carry 0.32 and 0.43×10^5 antigen sites, respectively (Fig 1). No binding occurred to Rh_{null} cells, which were devoid of Rh antigens. Dash erythrocytes (D--/D--), however, had two to three times more antigen sites (1.09×10^5) than did RBCs with a common phenotype. Erythrocytes Blo., which carry the D, c, E, and e antigens (probable genotype R^2R^0 as deduced from the family study, not shown) had 0.76×10^5 antigen sites, a value intermediate between R^1R^1 or R^2R^2 and D--/D--erythrocytes. The association constants of the antibody to R^1R^1 or R^2R^2 and D--/D-- erythrocytes were rather similar, $\sim 0.6 \times 10^8$ mol/L^{-1}, but were 10 times lower than with Blo. erythrocytes (6.7×10^8 mol/L^{-1}). Because Blo. erythrocytes also have a larger number of Rh(D) antigens than do cells with common phenotype and were readily available (from one of us, C.B.), they were used for further characterization and purification of the Rh(D) polypeptide.

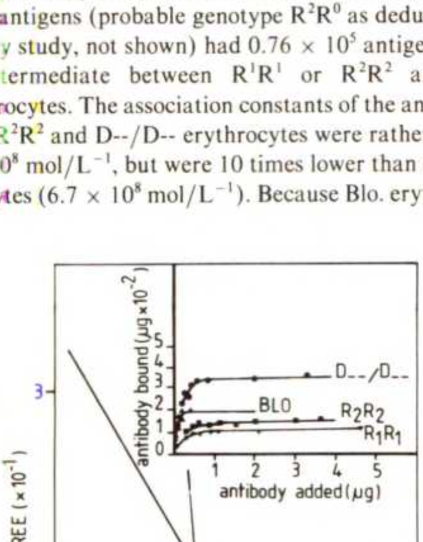

Fig 1. Binding of ^{125}I-labeled monoclonal anti-Rh(D) antibody to native human erythrocytes. Cells were incubated at room temperature with different amounts (0.017 to 6 µg) of ^{125}I-labeled IgG anti-Rh(D) as described in the Materials and Methods section. The amount of antibody specifically bound to Rh-positive RBCs was calculated from the difference of radioactivity uptake between Rh-positive (R^1R^1, R^2R^2, D--/D--, and Blo.) and Rh-negative (rr) RBCs. Only 0.06% to 10% of the total radioactivity was bound to Rh-negative cells in the range of antibody concentration used with nonspecific binding (ratio of cpm Rh-negative/cpm Rh-positive × 100) between 3.4% and 22%. Results plotted according to Scatchard.[31] D--/D--(●); BLO. cells (▲); R^2R^2 (■); and R^1R^1 (▼). Inset: saturation curves of erythrocytes by the monoclonal antibody.

Immune precipitation of the Rh(D) antigen. The immune complexes formed between the monoclonal antibody and the Rh(D) polypeptide from ^{125}I-labeled membranes were extracted by 5% Triton X-100 and isolated with a *S aureus* absorbent. The components eluted from this absorbent are shown in Fig 2, after analysis by SDS-PAGE. A band with an apparent molecular mass of 29-kd was clearly identified in all RBC samples from Rh-positive individuals (Fig 2, lanes 2, 3, and 4), whereas no material could be identified from Rh-negative (Fig 2, lane 5) or Rh_{null} (Fig 2, lane 6) RBCs therefore indicating that this component represents presumably the Rh(D) polypeptide. Much more material was immunoprecipitated from D--/D-- and Blo. erythrocytes (Fig 2, lanes 2 and 3) than from R^2R^2 RBCs (Fig 2, lane 4), correlating well with the mean number of Rh(D) antigen sites present on these cells. After gel electrophoresis, part of the radioactive material contained in the immune precipitates migrated as a light band of 58-kd (Fig 2, lanes 2 and 3) identified as the dimeric form of the 29-kd component following protein elution and reelectrophoresis (Fig 2, lanes 7 and 8).

Comparison of detergent-solubilized properties of the Rh(D) polypeptide and of the RBC membrane sialoglycoproteins. The distribution of the Rh(D) polypeptide

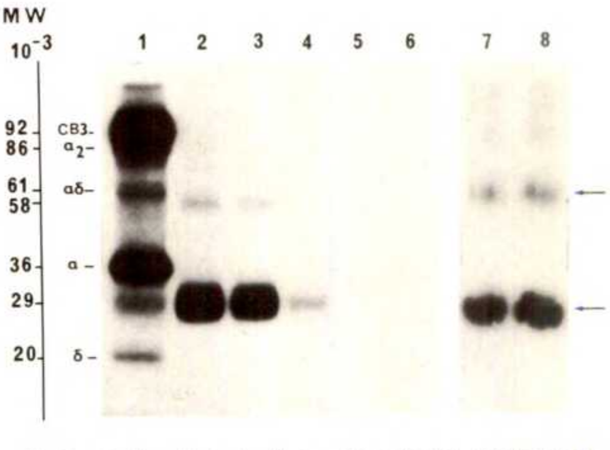

Fig 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of ^{125}I-labeled membrane components obtained following immune precipitation with the monoclonal anti-Rh(D) antibody. Electrophoresis was performed in 10% polyacrylamide gel in the discontinuous buffer described by Laemmli[34] under nonreducing conditions. Lane 1: profile of ^{125}I-labeled erythrocyte membranes (Blo.). Lanes 2 through 6: immune precipitates from D--/D--, BLO., R^2R^2, rr, and Rh_{null} erythrocytes. Lanes 7 and 8: reelectrophoresis of the 29-kd component obtained from the immune precipitates shown in lanes 2 and 3, respectively. Approximately 0.2% to 0.03% of the total ^{125}I-label incorporated into RBC membranes was immunoprecipitated from the R^2R^2 sample, whereas 3 to 4 times more material, respectively (0.05% to 0.06% and 0.08% to 0.1%) was associated with the 29-kd component from Blo. and D--/D-- RBCs. Nomenclature of RBC membrane sialoglycoproteins according to Anstee and co-workers.[38]

between the Triton X-100 soluble and insoluble membrane extracts was first examined using ^{125}I-labeled intact RBCs. The quantitative estimates were done by counting the radioactivity associated with the 29-kd band after SDS-PAGE of the Triton extracts. As shown in Table 1, the Rh(D) polypeptide was gradually solubilized from the membrane as the concentration of Triton X-100 increased. At low concentration of detergent, a significant part of the Rh(D) polypeptide was present in the insoluble residue. With 5% Triton X-100, however, $\leq$71% of the Rh material was solubilized. Because these results differ from those of other researchers,[16,17] the distribution of the Rh(D) antigen was also investigated following the binding of the ^{125}I-labeled anti-Rh(D) to Rh-positive and Rh-negative erythrocytes. The results obtained using saturating amounts of antibody were in agreement with those obtained above since ~86% of the Rh(D) immune complexes were solubilized by 5% Triton X-100 (Table 1). When the suspension of immune complexes and detergent (1:3 by vol) were centrifuged immediately, however, without shaking for 30 minutes at 10 rpm, ~80% and 61% of the radioactivity was found in the pellet obtained from the 1% and 5% Triton X-100 extracts, respectively (not shown).

For comparison, the distribution of the main RBC membrane sialoglycoproteins were investigated in parallel by staining the detergent soluble and insoluble extracts, prepared from ^{125}I-labeled membranes (including the shaking phase), with PAS after separation by SDS-PAGE (Table 1). More than 80% of glycophorin A was extracted by 1% Triton X-100 and almost 90% was removed from the membrane by 5% of detergent. Glycophorin A can be almost completely extracted by a high concentration of Triton X-100,[41,42] in marked contrast with glycophorin C which remains firmly bound to the detergent insoluble residue[43,44] even in the presence of 5% Triton X-100 (Table 1). Interestingly, glycophorin B was very easily solubilized from the membrane since ~70% of the glycoprotein was extracted by only 0.1% Triton X-100 and as much as 94.6% was removed by 1% of detergent (Table 1). Glycoprotein γ exhibited a behavior very similar to that of glycophorin C (Table 1), suggesting a strong association with the membrane skeleton.[45]

Purification and amino acid analysis of the Rh(D) polypeptide. The Rh(D) antigen readily loses its biological activity following extraction from the RBC membrane.[6,8] Therefore, the Rh(D) polypeptide was purified by immune precipitation of ^{125}I-labeled erythrocyte membrane preparations by the monoclonal anti-Rh(D) antibody. The immune complexes extracted as above by 5% Triton X-100 were separated by preparative PAGE, and the Rh(D) polypeptide was isolated by excision and electroelution of the 29-kd component. From 10 mL of Rh-positive membranes (Blo.), ~1 nmol of the Rh(D) polypeptide was obtained in a pure form as deduced from analytical SDS-PAGE (not shown). The purified preparation of Rh(D) polypeptide was virtually devoid of contaminating IgG (or IgG fragments), since there was no detectable band after electrophoretic transfer of the analytic SDS gel (5 μg loaded) to a nitrocellulose sheet[46] treated by a sensitive immunostaining procedure, using a peroxidase-labeled anti-human IgG, capable of detecting as little as 10 ng of monoclonal anti-Rh(D) (not shown). In another control experiment, we were unable to detect any Coomassie-blue stainable material on the analytical SDS gel using the same purification protocol with erythrocyte membranes from an Rh-negative donor (not shown). The presence of protein A and its fragments in the Rh(D) preparation was also investigated in a control experiment by studying the radioactive material released following heat elution of a ^{125}I-protein A–Sepharose matrix suspended in SDS buffer. The radioactivity released was very low and migrated on SDS gels as three light bands of 42, 27, and 23 kd (not shown). These components were absent from the Rh(D) polypeptide preparation purified by preparative gel electrophoresis by cutting out only the region around 29 kd. Assuming that Blo. erythrocytes carry 0.76 × 10^5 antigen sites (Fig 1), the purification yield of the Rh(D) polypeptide was ~7%. A similar purity and extraction yield was obtained with a preparation from D--/D-- RBCs (not shown). Attempts to increase the purification yield by extracting the membrane skeleton with alkali before immune precipitation were unsucesful. The amino acid composition of the Rh(D) polypeptide from Blo. erythrocytes is shown in Table 2. Two different preparations were analyzed. The small variations

Table 1. Solubilization of Glycoproteins and Rh Polypeptide From Human Erythrocyte Membranes by Triton X-100

Detergent Concentration (%, wt/vol)	Fraction Analyzed	Percentage of Material Solubilized Estimated by					
		cpm Counting		PAS Staining			
		Rh (29 kd)*	[^{125}I]anti-Rh$_0$(D)†	GPA	GPB	GPC	GP$_\gamma$
0.1	P	88	81	75.3	30.2	86	100
	S	12	19	24.7	69.8	14	
0.5	P	64	54	64.5	18.1	80	100
	S	36	46	35.5	81.9	20	
1.0	P	34	30	17.6	5.4	81.4	96
	S	66	70	82.4	94.6	18.6	4
5.0	P	29	14	11.5	7.4	80	70
	S	71	86	88.5	92.6	20	30

PAS, periodic acid-Schiff; GPA, GPB, GPC, glycophorins A, B, C; GP$_\gamma$, glycoprotein γ (or component E)[38,39] is also called glycophorin C by Furthmayr[40]. P, pellet; S, supernatant.

*Estimation of Rh(D) polypeptide from ^{125}I-labeled RBC membrane components after sodium dodecylsulfate-polyacrylamide gel electrophoresis.

†Estimation of Rh(D) polypeptide using the monoclonal ^{125}I-labeled anti-Rh(D) antibody.

Table 2. Amino Acid Composition of 29-kd Component Obtained From Rh-Positive Cells Blo.

Amino Acid	Preparation 1	Preparation 2
Cys-SO_3H	2.7	3.8
Asp	23.6	26.7
Me-SO_2	4.2	4.3
Thr	12.7	11.4
Ser	17.5	17.5
Glu	27.3	22
Pro	9.8	12
Gly	30	24.6
Ala	20.3	20.4
Val	18.1	19.8
Ile	13.3	11.8
Leu	21.8	24.3
Tyr	6.8	7.4
Phe	9.5	10.7
His	7.4	19
Lys	15.8	12
Arg	8.4	9.8

Results are expressed as mol of amino acid per mol of protein.

observed were presumably attributable to the presence of residual glycine from the electrophoretic buffer (preparation 1) and of sodium azide (preparation 2), which may have altered the aspartate and histidine determinations.[47] The results indicated that the Rh(D) polypeptide from these preparations contained several sulfhydryl residues and was composed of $\sim$250 amino acid residues.

DISCUSSION

We studied the binding properties of a human monoclonal anti-Rh(D) antibody produced by an EBV-transformed lymphoblastoid cell line[27a] to Rh-positive and Rh-negative human erythrocytes. This antibody was used to characterize some properties of the Rh(D) antigens and to purify the Rh(D) polypeptide to homogeneity.

The number of Rh(D) antigen sites of R^2R^2, R^1R^1 cells and of the rare D--/D-- erythrocytes estimated by the direct ^{125}I-labeling of the purified IgG fraction of the monoclonal antibody, correlated well with the results reported by others using conventional polyclonal human antisera.[4,5] No significant binding occurred, however, onto Rh-negative or Rh_{null} erythrocytes, which do not carry Rh(D) antigens, thereby confirming the strict blood group specificity of the monoclonal antibody and its potential use as reagent for routine Rh typing. The equilibrium constant of the monoclonal antibody was also determined using RBCs of known Rh(D) antigen site density. A value close to 0.6×10^8 mol/L^{-1} was calculated from the Scatchard plots drawn with R^2R^2, R^1R^1, and D--/D-- RBCs. This is in good agreement with most estimations calculated using human IgG anti-Rh(D) present in the sera obtained from 21 women at the time of delivery;[48] however, it is lower than the value found with the IgM monoclonal anti-Rh.[24]

The monoclonal antibody used here binds with high affinity (6×10^8 mol/L^{-1}) to Blo. erythrocytes which, with their large number of Rh(D) antigen sites (0.76×10^5), were selected for the further characterization and purification of the Rh(D) polypeptide. The large increase of equilibrium constant noted with Blo. erythrocytes was not related to their high antigen content, since the monoclonal antibody binds with a lower affinity (0.6×10^8 mol/L^{-1}) to D--/D-- RBCs, which carry even more Rh(D) antigens (Fig 1). Other studies of the Rh haplotypes segregating in family Blo., using a large collection of human polyclonal antisera, should indicate whether the RBCs from these donors express an unusual form of Rh complex.

A 29-kd component could be specifically immunoprecipitated from all Rh-positive cells investigated, whereas no material was isolated from Rh-negative or Rh_{null} RBC membranes. This was again in agreement with previous studies carried out with polyclonal antisera.[13,17] As suspected earlier,[15] a dimeric form (58 kd) of the 29 kd was also identified on the gels, but no component of higher molecular mass was present in the immunoprecipitates. Using different approaches, we found that as much as 70% to 80% of the Rh(D) polypeptide can be extracted from Rh-positive RBCs by 5% Triton X-100 (Table 1) under conditions in which glycophorins A and B were almost or completely extracted from the RBC membrane and glycophorin C as well as glycoprotein γ were firmly bound to the Triton-insoluble residue. These results suggest that only a small proportion of the Rh(D) polypeptide interacts significantly with the membrane skeleton. Gahmberg and Karhi[16] showed that 70% of the Rh(D) polypeptide was firmly anchored to the membrane skeleton. We also found that when the Triton X-100 extracts are prepared without shaking, 61% of the Rh(D) peptide is centrifuged with the 5% detergent insoluble residue. Up to 80% of this material was easily released after gentle shaking (10 rpm for 30 minutes), however, suggesting a loose association of the Rh(D) peptide with the membrane skeleton. These different results are clearly related to the method used for membrane solubilization, and only 60% to 72.7% of glycophorin A antigen–antibody complexes were extracted by 5% Triton X-100 in the study reported above.[16]

The Rh(D) polypeptide solubilized by 0.1% to 5% detergent could not be immunoprecipitated by the monoclonal antibody and did not bind to an affinity column prepared from the purified IgG monoclonal anti-Rh(D) antibody. The Rh(D) polypeptide was therefore purified on a large scale by a combination of immunoprecipitation and preparative PAGE using Blo. and D--/D-- RBC membrane preparations. The preparations obtained appeared homogeneous by usual criteria and had closely related amino acid compositions (Table 2), with a similar content of sulfhydryl groups. The yield of Rh(D) was, however, rather low ($\sim$7%), since the procedure could not be conducted with saturating amounts of anti-Rh(D) antibody. Indeed, from the binding studies reported above (Fig 1, inset), $\sim$5 to 10 mg of purified anti-Rh(D) antibody was deemed required for each mL of packed RBCs used during the purification to maximalize the yield. Using the present conditions, however, we obtained enough material for a partial characterization of the Rh(D) polypeptide and studies are now in progress to prepare a mouse monoclonal antibody against this protein, which would greatly facilitate its purification on a large scale for further biochemical analysis.

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Synergism Between Recombinant Growth Factors, GM-CSF and G-CSF, Acting on the Blast Cells of Acute Myeloblastic Leukemia

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The genes for the hemopoietic growth factors, GM colony-stimulating factor (CSF) and G-CSF have been cloned, and recombinant material is available for both. We tested these recombinant factors for their effects on the blast cells of acute myeloblastic leukemia (AML). Culture methods are available that support both colony formation by AML blasts and the growth of blast stem cells in suspension. Recombinant GM-CSF is active in both culture systems, although to a varying degree. We found that recombinant G-CSF was also effective; however, the two recombinant factors showed striking synergism for the stimulation of blast growth of cells from five of eight AML patients. In these cases, the combination was equivalent to the stimulating activity of supernatants from the continuous cell line 5637. This conditioned medium (HTB9-CM) is considered the standard for blast growth. Blasts from one of the patients grew without added factor. In another instance, recombinant GM-CSF alone was almost as effective as HTB9-CM. In the third case, both recombinant factors were active, but synergism was not observed and their combined effect was not equivalent to that of HTB9-CM. Both GM-CSF and G-CSF were active on normal bone marrow granulopoietic progenitors, but synergism was not observed. We conclude that the marked heterogeneity observed when AML blasts are examined by other criteria is also observed when their response to growth factors is evaluated.

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THE BLAST CELLS of acute myeloblastic leukemia (AML) proliferate in suspension cultures[1] or form colonies in cultures made viscid with methylcellulose[2] if a suitable source of growth factor(s) is included in the medium. This requirement has often been met using crude supernatants, either from phytohemagluttinin-stimulated leukocytes (PHA-LCM)[2] or a high factor-producing subclone of the continuous bladder carcinoma cell line 5637 (hereafter called HTB9-CM, a name used in the publication describing the clone and its active supernatants[3]). Recently, we[4] and others[5] showed that recombinant GM-CSF, first described as a requirement for granulocyte/macrophage colony formation in culture,[6] is an active growth factor for blast cells. The recombinant material was not as effective as HTB9-CM in promoting colony formation, however.[4]

G-CSF is a growth factor described originally as specific for granulopoiesis in culture.[7] Recently, molecular clones coding for this growth factor have been isolated[8,9]; their protein products proved to be effective stimulators not only of granulocyte progenitors, but also of other early hemopoietic cells. Recombinant G-CSF induced morphological differentiation in the murine continuous cell line WEHI3 and is reported to have a similar although less marked effect on freshly obtained human AML blast cells.[8]

We report the effects of recombinant G-CSF on AML blasts using clonogenic[2] and suspension culture assays.[1] We find that G-CSF is an effective stimulator of blast growth. Moreover, synergism occurs when GM-CSF and G-CSF are used together. In some instances, the effect of the combination of factors is the equivalent of HTB9-CM alone. Sensitivity to each factor, singly or in combination, however, varied from patient to patient.

MATERIALS AND METHODS

Cells. Normal marrow was obtained with informed consent from transplant donors. Peripheral blood was obtained with informed consent from eight patients with AML undergoing treatment at the Princess Margaret Hospital. The diagnosis was made on clinical and morphological grounds, using published criteria (French-American-British classification, FAB)[10] Table 1 summarizes the clinical data. Blasts were separated using the two-cycle Ficoll density-gradient separation procedure described earlier.[11] The cells were either placed in culture at once or preserved by freezing at −70°C in 50% fetal calf serum (FCS) and 10% dimethylsulphoxide (DMSO). Thawed cells were cultured in suspension containing 10% HTB9-CM for at least 3 days prior to plating in methylcellulose. Some experiments used cells that had been maintained continuously in suspension culture from 6 to 12 months (patients 5 and 8).

Cell culture methods. The clonogenic assay was done as described previously using HTB9-CM or recombinant growth factors.[2] Blast populations were cultured in microwells (Linbro/Titertek, Flow Laboratories, McLean, VA) in a medium consisting of α-minimum essential medium (MEM), 10% heat-inactivated FCS (growth medium) 0.8% methylcellulose and 10% HTB9-CM, or recombinant factors at various concentrations. The cultures were incubated for 7 days in a humidified atmosphere of 5% CO_2 in air at 37°C, after which time colonies containing >20 cells were counted with an inverted microscope. The secondary plating efficiency (PE2) was measured using the pooled colony technique described previously.[12] The cells were harvested from primary colonies, pooled, washed, and replated in microwells at a cell density of 2×10^4/well with media of the same composition used for the primary cultures. Colonies with >20 cells were counted after 7 days of incubation. PE2 was expressed as colonies per 10^4 cells plated. Blast cells were cultured in suspension in 1.7-cm tissue-culture wells (Linbro multiwell plates, Flow Laboratories).[3] As in the clonogenic assay, HTB9-CM (10%) or various concentrations of recombinant growth factors were used as a source of stimulation. The cells were suspended in growth medium at a concentration of 10^6/mL, with growth factor added. After 7 days, the cells were harvested and counted. The cells recovered from the suspensions were washed and replated in methylcellulose in microwells for blast colony formation as described

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Table 1. Clinical Characteristics of the Patient Population

Patient No.	FAB Classification	Age	Blasts in Peripheral Blood (%)	Outcome
1	M_4	67	90	Failure
2	M_4	40	60	Remission
3	M_4	56	57	Second remission
3b	M_4	56	53	Third remission
4	M_4	70	72	Partial remission
5	M_4	76	79	Remission
6	M_4	17	80	Remission
7	M_1	66	63	Failure
8	M_4	65	78	Remission

Blood was obtained at initial visit except for patient 3. Treatment consisted of high-dose cytosine arabinoside for induction and maintenance.[15]

earlier.[1] This procedure provided a measurement of the number of clonogenic cells in each dish that was recovered in suspension culture. The calculation consisted of multiplying the number of cells recovered in each dish by the plating efficiency in methylcellulose. Normal marrow was cultured after removing adherent cells by overnight incubation. The cultures consisted of 2×10^4 cells/well and growth medium with or without recombinant growth factors or HTB9-CM. Colonies were counted after 7 and 14 days in culture.

Recombinant growth factors. Purified recombinant GM-CSF was produced at the Genetics Institute (GI), Boston, from GM-CSF–transfected Chinese hamster ovary (CHO) cells. This material has a potency of 1,000 U/μg, where a unit is defined as the amount of factor needed to produce half-maximum colony formation in methylcellulose. Recombinant G-CSF was also made at GI. The published sequence of G-CSF[8,9] was used to prepare oligonucleotides. A mammalian expression cDNA library was prepared from RNA from TPA30-1, a SV40-transformed human trophoblast cell line, and was screened using these oligonucleotides as probes. Positive colonies were tested for biological activity by expressing the cloned cDNAs in COS cells and testing the supernatants using chronic myelogenous leukemia (CML) cells sensitive to growth factors (G. Wong and S. Clark, unpublished observations). The potency of these preparations was ~200 U/μL. In our experiments, supernatants were used from G-CSF–transfected COS cells; controls using supernatants from mock-transfected COS cells did not stimulate colony formation in excess of background. A result typical of these controls is shown in Fig 1.

Morphology. Cells were obtained either from colonies in methylcellulose or from suspension cultures, placed on coverslips by cytospin, and stained with Wright's stain. The cells were examined by light microscopy using oil immersion.

RESULTS

Activity of recombinant growth factors in methylcellulose. Because the source of G-CSF was an unpurified supernatant from transfected COS cells, it was tested on responsive blasts (patient 5) at a number of dilutions. The titration curve is shown in Fig 1 and compared with a supernatant from mock-transfected COS cells. Maximum activity occurred at a dilution of 10^{-3}, and the control COS supernatant was inactive.

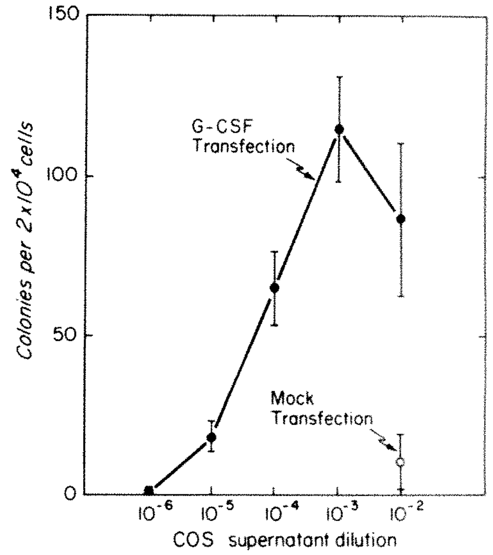

Fig 1. Titration curve for G-CSF in supernatants of transfected COS cells tested on blasts from the peripheral blood of patient 5. Each point is the mean of four determinations. SD is shown.

In the next series of experiments, GM-CSF and G-CSF were tested alone or in combination in methylcellulose. When used alone, G-CSF was usually a better stimulator of colony formation than was GM-CSF. Neither of the factors alone was equivalent to HTB-9, however. When the two recombinant factors were used together, synergism with respect to colony size and numbers was observed in five of eight patients. The augmentation of colony size is shown in Fig 2, a photomicrograph comparing cultures with G-CSF alone (panel B), GM-CSF and G-CSF in combination (panel D), GM-CSF alone (panel A), and HTB-9 (panel C). The two recombinant factors, combined in the same concentrations that gave maximum stimulation for each alone, supported the growth of colonies equivalent in size to those seen in cultures with HTB9-CM. As a further control, a supernatant from mock-transfected COS cells (dilution, 10^{-2}) was mixed with 2,000 pmol of GM-CSF and tested for its effect on colony formation in methylcellulose. No stimulation over that obtained with GM-CSF alone occurred, indicating that COS cells alone do not secrete an activity capable of a synergistic effect with GM-CSF.

The synergistic effect of the two recombinant growth factors on colony numbers is illustrated in Fig 3. Purified GM-CSF, ranging in concentration from 0.2 to 2,000 pmol/L, was tested for its effect on colony formation with and without the addition of G-CSF at a dilution of 10^{-2} or 10^{-3}. Colony formation with these dilutions of G-CSF alone are given at the left (Fig 3). Colony formation with 10%, 20%, and 30% HTB9-CM are shown at right. The synergism between the two recombinant factors is evident. Maximum stimulation by GM-CSF, or G-CSF alone, gave 11 ± 3 colonies/10^4 cells and 31 ± 4 colonies/10^4 cells, respectively. In combination, the highest tested concentrations of GM-CSF and G-CSF resulted in 92 ± 12 colonies/10^4 cells.

In addition to the synergistic effect, three other patterns of responsiveness to recombinant growth factors were observed. In one case, blast cells were independent of added stimulator whether in the form of HTB-9 CM or either of the recombinant factors. In a second case, nearly maximum colony

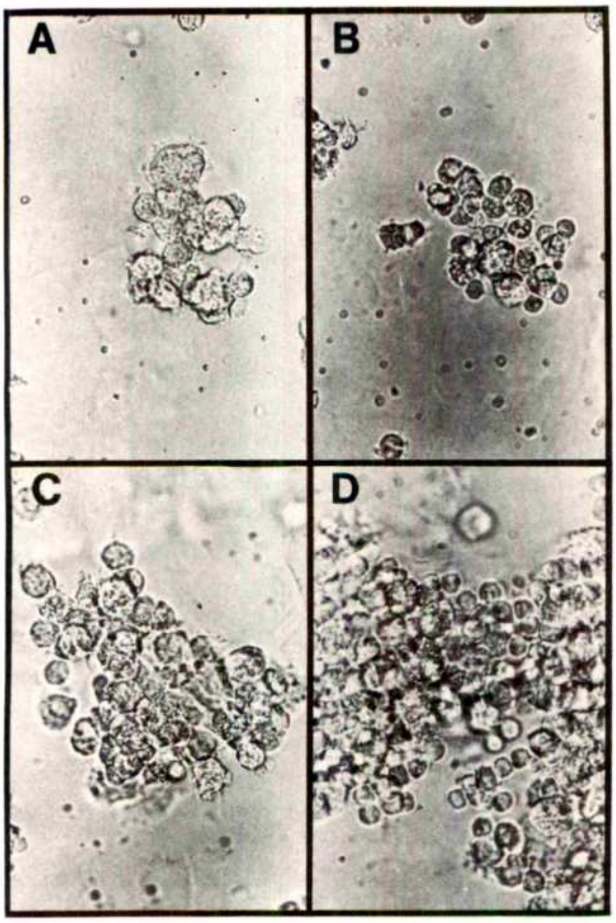

Fig 2. A photomicrograph of blast colonies formed with different growth factors. (A) G-CSF alone at a maximum stimulatory concentration of 2 nmol/L. (B) G-CSF alone at maximum stimulatory concentration of 200 U/mL. (C) 10% HTB-9 CM. (D) A mixture of GM-CSF and G-CSF, both at maximum stimulatory concentrations.

formation occurred with GM-CSF alone. The addition of G-CSF was additive, resulting in the same degree of stimulation as was obtained with HTB9-CM. In one other case, neither GM-CSF nor G-CSF was a very potent stimulator of growth in methylcellulose. The combination of the two factors was additive and not synergistic. These results are summarized in Table 2; colony formation with the various recombinant factors is shown normalized against growth with the effective stimulator HTB9-CM.

Two similar experiments were done with normal bone marrow cells, previously depleted of adherent cells. These were exposed to GM-CSF and G-CSF alone or in combination. In 7 days, when used alone, G-CSF stimulated more colony formation than GM-CSF (Fig 4). After 14 days, the number of colonies in culture with GM-CSF was approximately equal to that with G-CSF (data not shown). There was no evidence of synergism at either time in methylcellulose.

Activity of growth factors in suspension culture. Parallel experiments were done using the suspension culture method. Figure 5 shows a titration of GM-CSF, with or without the addition of G-CSF. The data are presented in the same form as the titration in methylcellulose shown in Fig 3 and include a comparison with HTB9-CM. Although both GM-CSF or G-CSF alone had small effects on the increase of clonogenic cells in suspension, a combination of the two was highly effective, yielding a maximum recovery exceeding that obtained with HTB9-CM.

Patient-to-patient variation observed with the suspension assay was similar to that which occurred with the methylcellulose assay. The data are presented in Table 3. Unfortunately, cells were not available from patient 6, the only representative of class 2. For the other classes, the growth responses were similar to those seen in methylcellulose.

Secondary plating efficiencies. Cells from blast colonies were replated in methylcellulose to measure PE2. The colonies were grown either directly from fresh or cryopreserved

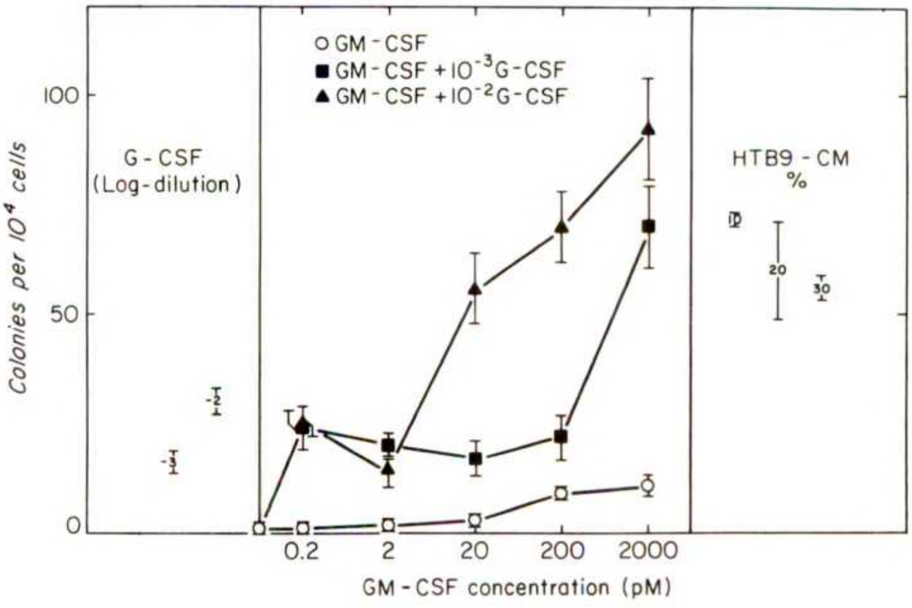

Fig 3. Center panel: titration of blast cells with recombinant GM-CSF alone or with two different concentrations of G-CSF, with colony formation as an endpoint. Left panel: values for G-CSF alone, with dilution of COS supernatants shown between error bars. Right panel: colony formation stimulated by HTB9-CM alone. SD is shown.

Table 2. Maximum Stimulation of Colony Formation by Recombinant Growth Factors

Class and Patient No.	GM-CSF*	G-CSF*	GM-CSF + G-CSF*
Class 1			
Patient 1	25.6	62.2	121
2	24.3	59.5	167.5
3	14.7	42.9	128
4	16.5	22.5	87.9
5	12.5	35.7	80.3
Class 2			
Patient 6	12.5	13.9	32.3
Class 3			
Patient 7	82.3	30	105
Class 4			
Patient 8		Factor independent	

*Values were normalized to HTB9-CM by the formula: n = no. of colonies with growth factor/no. of colonies with HTB9-CM × 100.

cells or after propagation of the cells in suspension. In each instance, the growth factor used in the early part of the experiment (colony formation only or suspension culture followed by colony formation) was included in the medium for the PE2 measurement. The data are shown in Table 4. Neither recombinant growth factor was as effective as HTB9-CM; but, as in other experiments, synergism occurred when both recombinants were included in the cultures. Indeed, the PE2 values were greater than those that occurred with HTB9-CM alone.

Morphology. Cells were examined by light microscopy under oil immersion after staining with Wright's stain. The predominant appearance was that of blast cells. For populations recovered from methylcellulose, numerous vacuoles were observed, presumably the result of ingestion of methylcellulose. From cells that had been exposed to G-CSF,

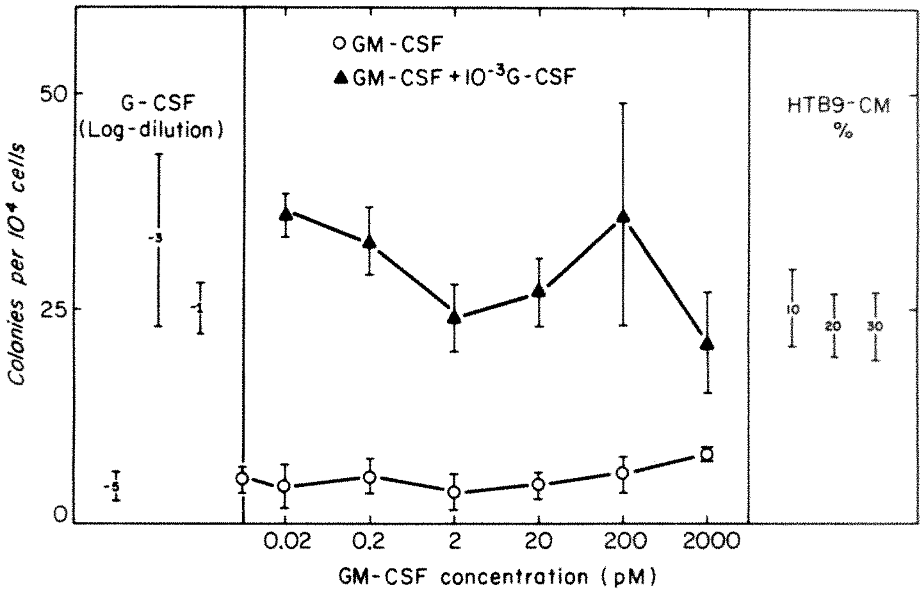

Fig 4. Center panel: titration of normal bone marrow cells with recombinant GM-CSF alone or with 10^{-3} dilution of G-CSF with colony formation as an endpoint. Left panel: values for G-CSF alone with dilution of COS supernatants shown between error bars. Right panel: colony formation stimulated by HTB9-CM alone. SD is shown.

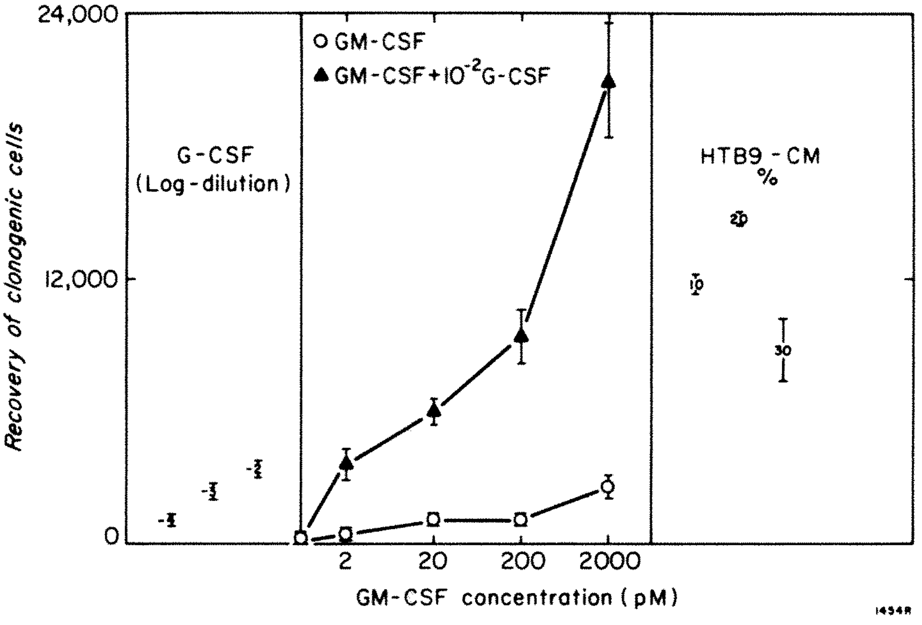

Fig 5. Effect of recombinant GM-CSF and G-CSF on growth of clonogenic cells in suspension. Left panel: values for G-CSF alone, with dilution of COS cells in suspension. Supernatants shown between error bars. Right panel: colony formation stimulated by HTB9-CM alone. SD is shown.

Table 3. Maximum Effect of Recombinant Growth Factors on Blast Progenitors in Suspension Cultures

Class and Patient No.	GM-CSF (%)*	G-CSF (%)*	GM-CSF + G-CSF (%)*
Class 1			
Patient			
3	8.1	14.8	312
3b†	18.1	22.9	142.9
4	1	2.2	58.7
Class 3			
Patient			
7	70	72	90
Class 4		Factor independent	
Patient			
8			

*HTB9-CM is 100%; values were normalized to HTB9-CM as in Table 1.

†A second sample from patient 3, taken 2 months after the first, after a brief remission.

occasional forms had band-shaped nuclei; in these cells, characteristic granules were not observed.

DISCUSSION

We showed that recombinant GM-CSF and G-CSF act synergistically on AML blast cells whether measured by the blast colony assay or in suspension; furthermore, similar effects occur on the PE2, which, like the suspension system, reflects blast stem cell renewal. Even though all but one of the patients are classified FAB M4, patient-to-patient variation is evident. For most patients (class 1), a combination of GM-CSF and G-CSF is as effective a stimulator as media conditioned by the continuous bladder cell line, 5637. Blasts from three patients responded to the growth factors in different manners. In one instance, both GM-CSF and G-CSF stimulated growth, but the effects were not synergistic and together did not approach the stimulation observed with HTB9-CM (class 2). Furthermore, in cultures with G-CSF, colonies were usually less compact than when HTB9-CM was used; indeed, even with HTB9-CM, colonies usually disperse after 7 days, and this tendency is marked with G-CSF alone.

This result suggests that yet other growth factors responsible for blast colony formation exist in HTB9-CM. In a second patient, GM-CSF alone was sufficient to give growth almost equivalent to that which occurred with HTB9-CM (class 3). Finally, the blasts from one patient were growth factor independent at the time of the initial study and thereafter when maintained in suspension (class 4). Thus, responses to these two growth factors show the patient-to-patient variation that has occurred when other biological parameters of AML blasts were measured.[13] The significance of this variability with respect to the biology of the tumor cells or clinical response is unknown.

The data may be considered in light of a model for the organization of the cellular populations in AML clones and the possible clinical significance of the model.[14] The blast population in AML is considered to be maintained independently by blast stem cells; these may either undergo self-renewal or pass through an event similar to determination, followed by a few terminal maturation divisions. These maturation steps may be considered to be part of differentiation. GM-CSF has been suggested to promote blast cell growth whereas G-CSF is a more potent differentiating agent. Indeed, in our experiments, some segmented forms were observed in cultures that had been exposed to G-CSF but not to GM-CSF. Care must be used, however, in assigning significance to such observations. An increase in the probability of determination as opposed to self-renewal may be a more important aspect of the differentiation process; it is certainly more relevant clinically since an increase in determination events would decrease the chance of survival of malignant clones. In our experiments, both GM-CSF and G-CSF were equally efficient in stimulating increases of blast clonogenic cells in suspension; recombinant GM-CSF was an effective stimulator of PE2 only in patient 7; neither recombinant factor was highly effective in this assay applied to other patients, although in combination they were usually more effective than HTB9-CM alone. Both growth in suspension and PE2 may be considered to reflect blast stem cell renewal; the data might be interpreted, therefore, as indicating that neither of the two recombinant factors affected the balance between self-renewal and determination in these populations of leukemic cells; any differentiation occurred only during postdeterministic divisions.

The synergism between GM-CSF and G-CSF observed in most AML blast populations was not observed in the two normal bone marrow specimens tested under conditions that would support granulocyte/macrophage colony-formation (Fig 4). For this limited sample population, it would not be appropriate to consider the data evidence for a leukemia-associated growth-factor response. Moreover, the cells of origins of such colonies (CFU-C) may not be comparable to blast stem cells. Resolution of these issues will require measurements on defined cell populations together with determination of receptor densities for both factors. Such studies are now in progress.

Table 4. Maximum Effects of Recombinant Growth Factors on Secondary Plating Efficiencies

	PE2 (Colonies/10^4 Cells)			
	Growth Conditions			
Cells	GM-CSF	G-CSF	GM-CSF + G-CSF	HTB9-CM
From suspension				
Patient 3b	19 ± 3	31 ± 10	86 ± 9	69 ± 8
4	0	0	42 ± 8	1 ± 0.5
7	336 ± 38	153 ± 21	197 ± 10	154 ± 18
From methylcellulose				
Patient 5	2 ± 0.5	1	47 ± 15	22 ± 5
3b	2 ± 0.5	9 ± 2	52 ± 8	37 ± 9

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Synergistic Inhibition of Platelet Activation by Plasmin and Prostaglandin I_2

By Andrew I. Schafer, George B. Zavoico, Joseph Loscalzo, and Ann K. Maas

Endothelial cell prostacyclin (PGI_2) inhibits platelet activation by raising platelet cyclic AMP. Previously, platelet activation was also shown to be blocked by plasmin formed by endothelium-derived tissue plasminogen activator (TPA). We have now studied interactions between PGI_2 and plasmin in the control of platelet function. PGI_2 and plasmin cause synergistic inhibition of thrombin- and ADP-induced aggregation of washed platelets. Inhibition by PGI_2 is similarly potentiated by TPA added to platelet-rich plasma to generate plasmin. Thrombin-stimulated rise in platelet cytosolic Ca^{2+}, measured by fura2 fluorescence, and thromboxane A_2 formation, measured by radioimmunoassay (RIA), are likewise synergistically inhibited by PGI_2 and plasmin. Plasmin neither increases nor potentiates PGI_2-stimulated increases in platelet cyclic AMP. Thus, PGI_2 and plasmin cause synergistic inhibition of platelet activation by both cyclic AMP-dependent and independent mechanisms. This interaction between two different endothelium-derived products may play an important role in localizing the hemostatic plug to a site of vascular injury by preventing further thrombin-mediated accrual of platelets.

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INTACT VASCULAR endothelium possesses antithrombotic properties that maintain blood fluidity under normal circumstances and prevent extension of the hemostatic plug beyond sites of vascular injury. Prostacyclin (prostaglandin I_2, PGI_2) formed by endothelial cells inhibits platelet activation by stimulating platelet adenylate cyclase and raising intracellular levels of cyclic AMP.[1,2] Tissue plasminogen activator (TPA) released from endothelial cells initiates fibrinolysis by converting plasminogen to the fibrinolytic protease plasmin. Plasmin not only has fibrin-digesting properties, but we and others previously showed that plasmin can also inhibit platelet activation in response to thrombin and other agonists by a noncyclic AMP-dependent mechanism.[3-6] We now report that PGI_2 and plasmin have synergistic inhibitory effects on platelet activation and propose that this interaction may have important physiologic consequences in the limitation of thrombus formation.

MATERIALS AND METHODS

Preparation of platelets. Venous blood anticoagulated with 13 mmol/L of sodium citrate was obtained from volunteers who had not ingested aspirin or other medications known to interfere with platelet function for at least 10 days before donation. Blood was centrifuged at 160 g for 10 minutes to obtain platelet-rich plasma (PRP). Platelets were separated from plasma by gel filtration by applying PRP to a column of Sepharose 2B. The platelets were eluted with a modified Tyrode's buffer containing 130 mmol/L NaCl, 9 mmol/L of sodium bicarbonate, 6 mmol/L of dextrose, 10 mmol/L of sodium citrate, 10 mmol/L of Trizma base, 0.8 mmol/L of KH_2PO_4, 3 mmol/L of KCl, 0.9 mmol/L of $MgCl_2$, 0.5% bovine serum albumin (BSA), pH 7.3. Platelet counts were determined in a counter (model F, Coulter Electronics, Hialeah, FL). Informed consent was obtained from all volunteers for blood donation with approval of the institutional Committee for the Protection of Human Subjects from Research Risks.

Platelet aggregation. Aggregation of PRP or gel-filtered platelets (following the addition of 3 mmol/L of $CaCl_2$) was monitored using a standard nephelometric method[7] in a dual-channel aggregometer (Payton Associates, Buffalo). For thrombin-induced aggregation in PRP, 0.5 mmol/L of glycyl-L-prolyl-arginyl-L-proline was included in all samples to prevent interference with the aggregation tracings by fibrin polymerization in plasma.

Platelet cytosolic ionized Ca^{2+}. Cytosolic ionized Ca^{2+} ($[Ca^{2+}]_i$) was monitored by fluorescence of washed platelets loaded with fura2-pentaacetoxymethyl ester (fura2/AM). PRP with 1 µmol/L of PGE_1 was titrated to pH 6.5 with acid-citrate-dextrose (NIH formula A) and centrifuged at 2,000 g for 10 minutes. The platelet pellet was resuspended in modified Tyrode's buffer as above except that KH_2PO_4 was replaced with 2 mmol/L of Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid); 0.1 µmol/L of PGE_1, 450 µg/mL of creatine phosphate, 100 µg/mL of creatine phosphokinase, and 1 µmol/L of fura2/AM were added, and the platelet suspension was incubated at 37°C for 45 minutes. Fura2/AM is hydrolyzed by nonspecific cytoplasmic esterases yielding the impermeant, Ca^{2+}-sensitive parent compound, fura2, as described by Grynkiewicz and colleages.[8] Fura2-loaded platelets were gel filtered to remove free fura2/AM. The platelet count was adjusted to a final concentration of 10^8/mL, and 1 mmol/L of $CaCl_2$ was added. For fluorescence measurements, 1.5 mL of platelet suspension was continually stirred at 37°C in a Fluorolog 2 spectrofluorometer (SPEX Industries, Edison, NJ) equipped with two excitation monochrometers and a chopper that quickly alternates (90 Hz) sample illumination by the excitation beam from each monochrometer. Excitation wavelengths were 340 and 380 nm, with a 5.4 nm bandpass. Emission was detected at 505 nm (4.5 nm bandpass) and collected in a SPEX DM1B Spectroscopy Laboratory Coordinator for calculation of the ratio of the fluorescence intensity at 340 and 380 nm. An increase in the ratio of 340/380 nm represents an increase in $[Ca^{2+}]_i$.

Thromboxane B_2 radioimmunoassay. The amount of thromboxane B_2 (TXB_2), the breakdown product of thromboxane A_2 (TXA_2), in supernatants of completed reactions was determined by standard radioimmunoassay (RIA) with phosphate-gelatin buffer (50 mmol/L of potassium biphosphate, 0.14 mol/L of NaCl, and 0.1% gelatin, pH 7.4), a 1:2,000 dilution of TXB_2 antiserum, and 10,000 dpm $[^3H]TXB_2$. After overnight incubation at 4°C, charcoal-coated dextran was used to separate antibody-bound from unbound radioligand.

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Cyclic AMP radioimmunoassay. Reactions were stopped by the addition of an equal volume of ice-cold 10% trichloroacetic acid and extracted four times with water-saturated ether. Cyclic AMP in platelets was measured by radioimmunoassay (cyclic AMP [^{125}I] RIA kit, New England Nuclear, Boston) and corrected for platelet counts.

Materials. The following reagents were obtained from commercial sources: fura2/AM from Calbiochem, La Jolla, CA; PGI_2, PGE_1, TXB_2, and TXB_2 antiserum from Upjohn, Kalamazoo, MI; [^{3}H] TXB_2 (100 to 150 Ci/mmol) from New England Nuclear, Boston; human thrombin from R.Q.P. Laboratories, South Bend, IN; ADP, aprotinin, and glycyl-L-prolyl-arginyl-L-proline from Sigma, St Louis; Sepharose 2B from Pharmacia Fine Chemicals, Uppsala, Sweden; and plasmin and S-2288 chromogenic substrate from Kabi, Stockholm. Recombinant human TPA was kindly provided by Genentech, South San Francisco.

Caseinolytic units (CU) of plasmin were defined according to Sgouris and colleagues[9]: 1 CU equals 1.14 Committee on Thrombolytic Agents (CTA) units.[10] Plasmin activity was determined in an S-2288 chromogenic substrate assay performed at 37°C in a spectrophotometer (Gilford 2400, Oberlin, OH). One CU plasmin equals ~0.45 nmol of plasmin. The purity of this human plasmin preparation was documented as previously reported[11]: when subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), it produced two major bands with apparent mol wt of 70,000 and 28,000 under reducing conditions and a single band with an apparent mol wt of 81,000 under nonreducing conditions.

RESULTS

Human platelets washed in a modified Tyrode's buffer were preincubated with either human plasmin for 10 minutes and/or PGI_2 for 3 minutes at 37°C, plasmin activity was neutralized by the addition of aprotinin, and platelet aggregation was initiated with either 0.2 U/mL of human thrombin or 10 μmol/L of ADP. As shown in Fig 1A, concentrations of plasmin and PGI_2 were determined that individually caused only slight inhibition of thrombin or ADP-induced aggregation. When platelets were preincubated with both plasmin and PGI_2 at the same concentrations as used individually, potentiation of the inhibitory effects was consistently noted. Similar experiments were performed with PRP instead of washed platelets: in this case, plasmin was generated in plasma by incubation with recombinant human TPA for 10 minutes instead of by the direct addition of plasmin. As shown in Fig 1B, preincubation of PRP with a combination of both TPA and PGI_2 at threshold inhibitory concentrations caused potentiation of inhibition of platelet aggregation in response to both thrombin and ADP.

To show that in platelets plasmin and PGI_2 exert their synergistic inhibitory effects not only on the aggregation response itself but also on the transduction of extracellular signals, we performed experiments to determine the interactions between plasmin and PGI_2 on platelet Ca^{2+} mobilization and the release and metabolism of arachidonic acid induced by thrombin. As shown in Fig 2, preincubation of fura2/AM-loaded platelets with either 1.0 nmol/L of PGI_2 for 3 minutes alone or 0.025 CU/mL of plasmin for 10 minutes alone at 37°C caused only a slight inhibition of thrombin-induced elevation in $[Ca^{2+}]_i$. When platelets were preincubated with both PGI_2 and plasmin at the same concentrations, however, marked inhibition of thrombin-induced Ca^{2+} mobilization occurred. Further addition of a fourfold higher concentration of thrombin did not overcome this synergistic inhibition by the combination of PGI_2 and plasmin.

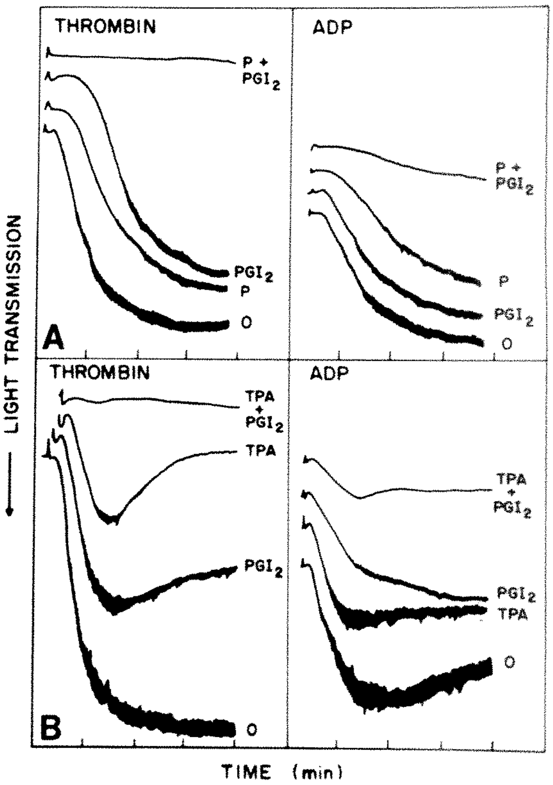

Fig 1. Synergistic inhibition of thrombin- and ADP-induced platelet aggregation (A) by prostaglandin I_2 (PGI_2) and plasmin in washed platelets, and (B) by PGI_2 and tissue plasminogen activator (TPA) in platelet-rich plasma. (A) Washed platelets were preincubated at 37°C for 10 minutes with buffer blank (O) or plasmin (P). Buffer blank or PGI_2 was added for the last 3 minutes of the preincubation; plasmin was then inactivated with aprotinin. Platelets were stimulated with either 0.2 U/mL of human thrombin or 10 μmol/L of ADP. Concentrations of plasmin and PGI_2 in the combined incubations were the same as those used individually. Final concentrations of plasmin and PGI_2 were 0.25 caseinolytic units (CU)/mL and 3 nmol/L, respectively, for thrombin-induced aggregations, and 0.2 CU/mL and 0.1 nmol/L, respectively, for ADP-induced aggregations. (B) PRP was preincubated at 37°C for 10 minutes with buffer blank (O) or recombinant human TPA. Buffer blank or PGI_2 was added for the last 3 minutes of the preincubation, and platelets were activated with either thrombin or ADP, as described above. Concentrations of TPA and PGI_2 in the combined incubations were the same as those used individually. Final concentrations of TPA and PGI_2 were 40 μg/mL and 1 nmol/L, respectively, for thrombin-induced aggregations, and 4 μg/mL and 2 nmol/L, respectively, for ADP-induced aggregations.

Because thrombin-activated phospholipase C stimulates the release of endogenous arachidonic acid, which is metabolized to the potent proaggregatory and vasoconstrictor eicosanoid TXA_2 in platelets, we studied the effects of plasmin and PGI_2 on inhibition of thrombin-stimulated TXA_2 formation, measured by radioimmunoassay of its breakdown product, TXB_2. As shown in Table 1, concentrations of plasmin and PGI_2 were determined that individually caused only slight inhibition of thrombin-induced TXB_2 production.

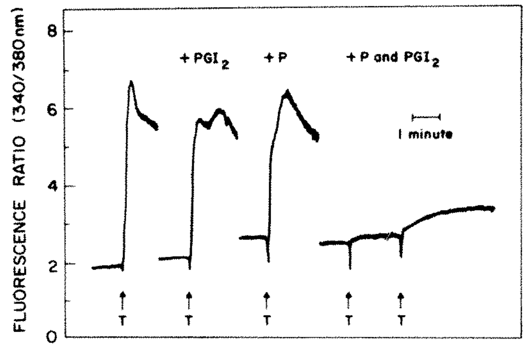

Fig 2. Synergistic inhibition of thrombin-induced increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by plasmin and prostaglandin I_2 (PGI_2) in washed platelets. Tracings show fura2 platelet $[Ca^{2+}]_i$ by fura2 fluorescence in response to the addition of 0.25 U/mL thrombin (T) at the point of each arrow. From left to right individual tracings show: no preincubation; preincubation with 1 nmol/L of PGI_2 for 3 minutes; preincubation with 0.025 caseinolytic units (CU)/mL of plasmin for 10 minutes; preincubation with 0.025 CU/mL of plasmin for 10 minutes plus 1 nmol/L of PGI_2 for 3 minutes. In the last tracing, an additional stimulus of 1 U/mL T was introduced 3 minutes after the initial stimulus of 0.25 U/mL of T.

When platelets were preincubated with a combination of both plasmin and PGI_2 at the same concentrations, marked potentiation of the inhibitory effect on TXB_2 formation was noted. Although the synergism between the inhibitory effects of plasmin and PGI_2 on platelet aggregation, $[Ca^{2+}]_i$, and TXB_2 production was reproducibly and consistently observed, as yet unexplained individual variations in the magnitude of these platelet responses were found.

The potentiating effect of plasmin on inhibition of platelet activation by PGI_2 was not mediated by cyclic AMP. As shown in Table 2, plasmin did not increase platelet cyclic AMP content. Furthermore, plasmin did not potentiate the PGI_2-induced elevation of platelet cyclic AMP levels.

DISCUSSION

Our studies indicate that plasmin and PGI_2 act synergistically to inhibit signal–response coupling in platelets. PGI_2 exerts this effect by raising the intracellular concentration of cyclic AMP. The mechanism of platelet inhibition by plasmin is different and possibly more complex. Loss of platelet membrane glycoproteins following plasmin treatment[12,13] might lead to loss of platelet receptors for thrombin[14,15] and other agonists. We also recently showed that plasmin can activate platelet protein kinase C.[11] Activation of protein kinase C by plasmin, detected by phosphorylation of the 47-kDa protein in $^{32}PO_4$-labeled platelets as previously described,[11] did occur under the conditions of these experiments (data not shown). Activation of platelet protein kinase C, in turn, feeds back to inhibit extracellular signal transduction[16,17] in response to thrombin and other agonists. Therefore, plasmin appears to exert its inhibitory effects on platelets, at least in part, by activation of protein kinase C. These results suggest that cyclic AMP and protein kinase C activation may synergistically inhibit the transduction of extracellular signals in platelets. This synergistic effect may be mediated by protein kinase C-induced phosphorylation and inactivation of the inhibitory guanine-nucleotide-binding regulatory component (N_i protein) of platelet adenylate cyclase.[18,19]

Thus, PGI_2 and plasmin act synergistically to inhibit platelet activation by blocking Ca^{2+} mobilization and the release and metabolism of arachidonic acid by both cyclic AMP and noncyclic AMP-mediated mechanisms. The potentiating inhibitory actions of plasmin and PGI_2 on platelet activation described in this study may operate in vivo as an important antithrombotic mechanism. Thrombin generation at the site of vascular injury stimulates both fibrin formation and platelet aggregation to form a hemostatic plug. Surrounding the focus of injury, however, PGI_2 and TPA are released from intact endothelial cells; thus, PGI_2 and plasmin, possibly generated at locally amplified concentrations, may act synergistically as potent inhibitors of further thrombin-mediated accrual of platelets to the hemostatic plug beyond the site of vascular injury.

Table 1. Potentiation of Inhibition of Thrombin-Induced Platelet TXA_2 Production by Plasmin and PGI_2

Preincubations	TXB_2 (pg/10^6 Platelets)
Control	196.0 ± 6.3
Plasmin	150.8 ± 5.7
PGI_2	149.8 ± 25.6
Plasmin + PGI_2	26.4 ± 0.8

TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; PGI_2, prostaglandin I_2; CU, caseinolytic unit.

Washed platelets (1.5 × 10^8/mL) were preincubated at 37°C for 10 minutes with buffer blanks (control), with 0.2 CU/mL of plasmin for 10 minutes, with 10^{-8} mol/L of PGI_2 for 3 minutes, or with a combination of 0.2 CU/mL of plasmin for 10 minutes and 10^{-8} mol/L of PGI_2 for 3 minutes. Aprotinin was added to all samples following the preincubation periods, and platelets were then activated with 0.37 U/mL of thrombin for 3 minutes. TXB_2 was measured by radioimmunoassay of supernatants of the completed reactions. Data are means ± SE of triplicate determinations of two separate experiments.

Table 2. Effects of Plasmin and Thrombin on PGI_2-Stimulated Platelet Cyclic AMP

Additions	Cyclic AMP (pmol/10^9 Platelets)
Control	96.0 ± 16.1
Thrombin	123.5 ± 17.7
Plasmin	124.6 ± 17.5
Plasmin + thrombin	118.2 ± 20.2
PGI_2	837.4 ± 26.5
PGI_2 + thrombin	373.1 ± 19.4
Plasmin + PGI_2	871.9 ± 34.7
Plasmin + PGI_2 + thrombin	569.4 ± 24.7

Abbreviations as in Table 1.

Platelets washed by gel filtration (~1.5 × 10^8/mL) were preincubated at 37°C for 10 minutes with buffer blank (control) or plasmin (0.25 CU/mL). Buffer blank or PGI_2 (10^{-8} mol/L) were then added for 30 seconds, followed by buffer blank or thrombin (0.75 U/mL) for an additional 2 minutes. Reactions were stopped by the addition of an equal volume of ice-cold 10% trichloroacetic acid. Cyclic AMP in platelets was measured by radioimmunoassay and corrected for platelet counts. Data are means ± SE of quadruplicate determinations of three separate experiments.

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Activities of Four Purified Growth Factors on Highly Enriched Human Hematopoietic Progenitor Cells

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The activities of four purified human growth factors: biosynthetic (recombinant) granulocyte-macrophage colony-stimulating factor (GM-CSF); recombinant erythroid-potentiating activity (EPA); natural and recombinant pluripoietin (Ppo); and natural pluripoietin alpha (Ppo α), were compared on the growth of hematopoietic colonies from enriched populations of human marrow and blood progenitor cells. Conditioned medium from the Mo T cell line (MoCM) was used as a standard positive control. We found that activities of GM-CSF and Ppo α on the growth of hematopoietic colonies were indistinguishable; Ppo α is now believed to be identical to GM-CSF. Both factors were able to promote the growth of colonies derived from subpopulations of CFU-GM, BFU-E, and CFU-GEM. Colonies derived from CFU-GM and CFU-GEM in cultures stimulated by GM-CSF and Ppo α were much smaller than in cultures stimulated by MoCM. In contrast to previous reports in which less highly enriched progenitors were used as target cells, Ppo had no detectable activity on the growth of colonies derived from BFU-E or CFU-GEM but promoted the growth of a subpopulation of CFU-GM derived colonies. Ppo is now recognized to be identical to G-CSF. The GM colonies in cultures stimulated by G-CSF (Ppo) were much smaller than in cultures stimulated by MoCM. EPA had no detectable activity on either the size or number of colonies derived from CFU-GM, BFU-E, or CFU-GEM. Results from experiments using target cell populations of marrow fractions separated by velocity sedimentation and marrow populations following freezing suggested that GM-CSF (Ppo α) and G-CSF (Ppo) primarily affect the growth of relatively mature subpopulations of progenitor cells. It is clear from these results that additional factor(s) are present in MoCM that are necessary to stimulate CFU-GM, BFU-E, and CFU-GEM maximally in vitro.

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SEVERAL purified growth factors with demonstrable activity in stimulating the growth of human hematopoietic progenitor cells recently became available. To examine the direct action of these growth factors on progenitor cells properly, several essential conditions must be established: (a) The target cells must be highly enriched populations of progenitors depleted of accessory cells that may endogenously produce stimulating activities or mediate the action of exogenous growth factors; (b) the number of colonies per culture should be kept low to avoid indirect stimulation produced by developing colonies (ie, large numbers of developing CFU-GM derived colonies may potentiate the survival of BFU-E derived colonies); (c) the semisolid culture environment must be sufficiently dense to avoid migration of cells that could result in overlapping colonies; (d) the activities of the purified growth factors must be compared on target populations from several different donors because the composition of progenitor cell populations vary from donor to donor (eg, number and size of colonies, morphological composition of CFU-GM derived colonies, and relative maturity of progenitor cells).

Using these conditions, we compared the activities of four purified growth factors. Human granulocyte-macrophage colony-stimulating factor (GM-CSF)[1] and erythroid-potentiating activity (EPA)[2] were purified from serum-free Mo-conditioned medium (MoCM) prepared from the Mo T cell line.[3] GM-CSF, a glycoprotein with a mol wt of 22,000, has been reported to: (a) stimulate the growth of colonies derived from human granulocyte-macrophage progenitors (CFU-GM)[1]; (b) stimulate colony formation by the KG-1 myeloid leukemia cell line[1]; (c) inhibit neutrophil motility (NIF-T activity)[1]; and (d) activate neutrophils (NAF).[4] Human GM-CSF was subsequently cloned from an expression complementary DNA library prepared from Mo cell messenger RNA.[1,5] The recombinant GM-CSF was expressed in Cos (monkey) cells, and the supernatant was assayed. A single human gene was demonstrated to encode a protein with NIF-T,[1] CSF,[1] and NAF[4] activities. Recently, recombinant GM-CSF was shown to have burst-promoting activity (BPA) for erythroid burst-forming units (BFU-E)[6-11] and multipotential colony-stimulating activity for CFU-GEMM[6,7,9,10] from enriched populations of human hematopoietic progenitor cells.

EPA has a mol wt of 28,000 and has been reported to stimulate the growth of both early and late erythroid precursors from buffy coat and light-density human bone marrow cells, as well as colony formation by the K562 human erythroleukemia cell line.[2] Subsequently, a complementary DNA molecular clone that encodes EPA was isolated, and the recombinant EPA was expressed in Cos cells and Chinese hamster ovary (CHO) cells.[12] More recently, the structure of a human tissue inhibitor of metalloproteinases (TIMP), a protein identical to EPA, was described.[13] Whether TIMP has erythroid-potentiating activity has not yet been determined.[13]

Pluripoietin (Ppo)[14] and pluripoietin alpha (Ppo α)[15] are

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human hematopoietic growth factors purified from the 5637 human bladder carcinoma cell line conditioned medium which has previously been shown to produce hematopoietic growth factors.[16,17] Ppo has a mol wt of 18,000 and has been reported to: (a) support the growth of colonies derived from human CFU-GEMM, BFU-E, and CFU-GM from low-density, nonadherent and T lymphocyte-depleted human bone marrow cells[14]; (b) induce differentiation of the human promyelocytic leukemic cell line HL-60 and the murine myelomonocytic leukemic cell line WEHI-3B (D+)[14]; (c) induce proliferation of low-density, nonadherent and T cell-depleted or MY10-positive human bone marrow cells in liquid culture[18]; (d) activate the function of peripheral blood granulocytes (ADCC) and monocytes[18]; (e) synergize with monocytes in the interleukin-2 (IL-2)–induced stimulation of lymphokine-activated killer (LAK) activity[18]; (f) regulate the expression of the MY10 antigen on bone marrow cells[18]; and (g) synergize with Ppo α in supporting the growth of fresh myeloid leukemic cells.[18] The identity of the purified protein (Ppo) has been confirmed by molecular cloning of the gene.[19]

Ppo α has a mol wt of 16,500 and has been reported to: (a) support the growth of colonies derived from human CFU-GEMM, BFU-E, and CFU-GM from low-density, nonadherent and T lymphocyte-depleted human bone marrow cells[15]; (b) induce differentiation of the human promyelocytic leukemic cell line HL-60[15]; (c) induce expression of chemotactic peptide receptors in leukemic cells as well as in normal peripheral blood granulocytes[15]; and (d) function as a chemoattractant for neutrophils and inhibit their migration.[15]

The activities of these four purified growth factors were studied on enriched normal human hematopoietic progenitor cells. Using density separations and a panel of monoclonal antibodies followed by immunoadherence (panning) or immune rosetting, we depleted normal bone marrow and peripheral blood specimens of accessory and maturing cells to obtain enriched populations of progenitor cells by negative selection. In some studies, postpanning marrow progenitor cells were further enriched by velocity sedimentation in a Ficoll isokinetic gradient,[20-24] which separates cells primarily on the basis of cell size. As we[24] and others[25-44] have previously found, progenitor cells differing in size also differ in degree of maturation, with the most primitive progenitor cells residing primarily in the small cell fractions and the more mature progenitor cells residing primarily in the larger cell fractions. We therefore used the isokinetic gradient to determine the degree of maturation of the progenitor cells stimulated by the purified growth factors. In addition, postpanning marrow cells, either before or after velocity sedimentation separation, were studied after being frozen to determine the activities of the purified growth factors on progenitors that had survived freezing.

MATERIALS AND METHODS

Bone Marrow Specimens

After appropriate human protection committee validation and informed consent, posterior iliac crest bone marrow aspirations were obtained from normal volunteers. The mean cell count of the undiluted marrow aspirates was 61×10^6 nucleated cells/mL. Buffy coat (BC) cells were collected after centrifugation of the undiluted marrow aspirates at 800 rpm for 10 minutes at 5°C and suspended in McCoy medium supplemented with 10% fetal calf serum (FCS) (Sterile Systems Inc, Logan, UT). The diluted BC cells were first depleted of platelets by centrifugation over Percoll (1.050 g/cm^3) (Pharmacia Fine Chemicals, Piscataway, NJ) at 2,000 rpm for 15 minutes at 5°C. All the supernatant containing the platelets was discarded, and the pellet of BC cells was recovered. We have found that this procedure results in maximal removal of platelets, which is essential to avoid clumping in our final enriched fraction. Light-density cells were subsequently obtained by centrifugation over Percoll (1.075 g/cm^3)[45] at 2,000 rpm for 15 minutes at 5°C. The mean recovery of the initial total number of nucleated cells following centrifugation to obtain the BC cells and sedimentation on Percoll (1.050 g/cm^3) was 81%; it was 42% following sedimentation on Percoll (1.075 g/cm^3).

Blood Specimens

After appropriate human protection committee validation and informed consent were obtained, heparinized peripheral blood specimens (150 to 200 mL) were obtained from normal volunteers. Light-density (density <1.077 g/cm^3) mononuclear cells (MNCs) were obtained by sedimentation of diluted whole blood on a Ficoll-Hypaque (Pharmacia) density cushion.[46] MNCs were depleted of most monocytes by adherence to plastic tissue culture dishes for 40 minutes at 37°C before incubation with a panel of monoclonal antibodies (Mabs).

Monoclonal Antibodies

Ascites fluids containing Mabs anti-B1,[47] anti-B4,[48] anti-My4,[49] anti-My8,[49] anti-N901,[50] anti-903 (directed against the same antigen as anti-Mo1 but an IgG1 Mab) (J.D. Griffin, personal communication),[51] and anti-I2[52] were kindly supplied by Dr James D. Griffin and Dr Stuart F. Schlossman (Dana Farber Cancer Center, Boston). Anti-human LyT-3 (clone 9.6) Mab[53] was initially obtained from New England Nuclear (Boston), but more recently has been kindly supplied by Dr John A. Hansen (Fred Hutchison Cancer Center, Seattle). Ascites fluid containing anti-glycophorin A (R10) Mab[54] was kindly supplied by Dr Paul Edwards (Ludwig Institute for Cancer Research, Surrey, England). Anti-Leu-1[55,56] and anti-Leu-4[56] in the form of tissue culture supernatants of cloned lines were kindly supplied by Dr Robert L. Evans (Sloan-Kettering Institute for Cancer Research, New York). Anti-Leu-9[57,58] was obtained from Becton Dickinson Monoclonal Center (Mountain View, CA). WEM-G11 Mab[59] was supplied through the courtesy of Dr Donald Metcalf by Dr Angel Lopez (Institute of Medical and Veterinary Science, Adelaide, South Australia).

Enrichment of Bone Marrow Progenitor Cells by Negative Selection

Panning procedure. Affinity plates for panning were prepared 2 to 4 days prior to use by the indirect method of Barker and colleagues[60] as previously described in detail.[61] One hundred to 200×10^6 light-density bone marrow cells were incubated with saturating amounts of a panel of Mabs (anti-B1, anti-B4, anti-My4, anti-My8, anti-903, anti-N901, anti-human LyT-3, anti-Leu-1, and anti-glycophorin A) for 30 minutes on ice. After coating, the cells were washed twice with ice-cold McCoy's 5A medium + 10% heat-inactivated (H-I) FCS and adjusted to a cell concentration of 2×10^6/mL in the same medium. WEM-G11, which results in further depletion of promyelocytes and myelocytes, was not obtained until recently and therefore was only included in the panel of Mabs in a few of the most recent studies. The panning procedure was performed as previously described.[61]

Representative antibody-negative populations were examined by fluorescent activated cell sorter (FACS) analysis and were negative for all Mabs used in the negative selection procedures, thus demonstrating the complete removal of all known accessory cell populations.

A mean of 1.9%, and more recently with the addition of WEM-G11, 0.5%, of the initial buffy coat cells were recovered in the nonadherent antibody-negative fraction. The mean recoveries of progenitor cells from the light-density fraction were 47% for BFU-E (35% to 63%) and 68% for day 14 CFU-GM (52% to 88%). Because none of the antigens identified by the monoclonal antibodies used in the depletion procedures are present on hematopoietic progenitor cells, the loss of progenitor cells most likely reflects nonspecific loss.

Velocity Sedimentation in an Isokinetic Gradient

A modification of the isokinetic gradient of Pretlow and colleagues[20-23] for velocity sedimentation was used to separate the nonadherent antibody-negative cells postpanning primarily on the basis of differences in cell size as previously described.[24] McCoy's 5A medium (modified) supplemented with 10% FCS and 25 mmol/L of Hepes buffer was used to prepare all solutions used in the gradient. The gradient was constructed in an IEC (International Equipment Company, Needham Heights, MA) 100-mL capacity polycarbonate tube placed on ice. Thirty-five milliliters each of 2.7% and 5.5% (wt/vol) Ficoll were introduced into a GM-1 gradient mixer (Pharmacia). The solution was allowed to flow through tubing connected to a Gilson Minipuls II peristaltic pump (Gilson Medical Electronics, Middleton, WI) at 2 mL/min and layered on a 5.5-mL cushion of 30% (wt/vol) Ficoll. Five milliliters of cell suspension were then layered on the top of the gradient. The gradient tube was slowly brought up to 780 rpm in an IEC refrigerator centrifuge (model PR-2) at 4°C and centrifuged for 15 minutes. After centrifugation, the gradient tube was placed on ice and a 15-gauge needle, equipped with a custom-made conical brass cap, was inserted through the center of the gradient. A 35% solution of sucrose was pumped through the long needle at ~3 mL/min, and 4-mL fractions were collected by upward displacement of the gradient. The upward displacement method minimizes the skewing effect commonly observed when fractions are collected through a hole made in the bottom of the gradient tube. In contrast to sedimentation at unit gravity, this system uses a centrifugal field, permitting the separation to utilize a much longer gradient and to be completed in a short period of time.

Cryopreservation

Bone marrow cells were cryopreserved using a mixture containing both 5% dimethylsulfoxide (DMSO) and 6% hydroxyethyl starch (HES) as previously described.[62] In addition, the cell suspension contained 20% to 45% autologous plasma or serum. Cells were placed in Nunc polypropylene cryotubes (Vangard International, Neptune, NJ) and initially were frozen at −120°C in a Revco freezer (Rheem, Asheville, NC) and later were transferred to a Union Carbide liquid nitrogen refrigerator (Union Carbide, Indianapolis). When defrosting, the cell suspension was immediately added to McCoy's medium supplemented with Hepes buffer, 10% FCS, and 50 to 60 U of deoxyribonuclease I (DNase) (Cooper Biomedical, Malvern, PA). The cells were incubated with DNase for 30 minutes on ice and then layered on a Ficoll-Hypaque density cushion. Following centrifugation, the viable cells were recovered at the Ficoll-Hypaque–plasma interface.

Freezing of postpanning marrow cells leads to further enrichment of progenitor cells since freezing destroys more mature progenitors, including myelocytes, the major contaminating cells after panning. The mean percentage of blasts following freezing was 77% (53% to 100%). The mean recoveries of progenitor cells after freezing were 49% for BFU-E (20% to 79%) and 39% (10% to 71%) for day 14 CFU-GM.

Enrichment of Peripheral Blood Progenitor Cells by Immune Rosetting

Negative selection. Peripheral blood light-density, nonadherent cells were incubated with optimal concentrations of a panel of Mabs (anti-B1, anti-B4, anti-My4, anti-My8, anti-903, anti-N901, anti-human LyT-3, anti-Leu-1, anti-Leu-4, and anti-Leu-9) for 30 minutes on ice. After being coated, the cells were washed twice and resuspended in McCoy medium containing 10% H-I FCS to a cell concentration of 15 × 10^6 cells/mL. One milliliter of cell suspension was mixed with 1 mL of a 2% solution of bovine RBCs (Hazleton, Denver, PA) coated with affinity-purified goat anti-mouse IgG (Boehringer Mannheim, Indianapolis) by $CrCl_3$ linkage.[63] After centrifugation, the tubes were placed on ice for 30 minutes, at which time the pelleted cells were resuspended and rosetted and nonrosetted cells were separated by centrifugation on a Percoll density solution (1.080 g/cm^3). The nonrosetted antibody-negative cells were recovered at the interface and washed twice with complete medium.

Positive selection for further enrichment. In some studies, the nonrosetted antibody-negative cells were incubated with anti-I2 and immune rosetted as described above. The pelleted rosetted cells were recovered and, after lysis of indicator bovine RBCs with Tris-buffered NH_4Cl, the Ia positive cells were washed twice with complete medium.

Progenitor Cell Assays

To assay for colonies derived from BFU-E, CFU-GM, and CFU-GEM, the enriched blood and bone marrow progenitor cell populations were cultured in methylcellulose as previously described.[64,65] Cell cultures consisted of 1 mL of Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Grand Island, NY) containing 24% FCS, 0.8% deionized bovine serum albumin (BSA) (Sigma Chemical, St Louis), 10^{-4} mol/L of 2-mercaptoethanol (Sigma), and methylcellulose at a final concentration of 1.3% in 35-mm Lux tissue culture dishes (Miles Scientific, Naperville, IL). The increased methylcellulose concentration results in large, compact colonies, which facilitates accurate scoring. One unit of partially purified human urinary erythropoietin (EPO) (specific activity 49 U/mg) (Toyobo, NY) was added either at the initiation of culture or 48 hours later.

Positive control cultures to measure the optimal cloning potential of the enriched progenitors contained 10% of a selected lot of MoCM prepared from the human Mo T lymphoblast cell line.[3,66] IMDM was substituted for MoCM in negative control cultures. The purified growth factors were added at appropriate concentrations as described in the Results section.

In some studies, bovine hemin (ferric chloride protoporphyrin I ×) (Sigma) was added at a final concentration of 0.2 mmol/L.[67,68] The addition of hemin results in larger and more clearly hemoglobinized BFU-E with MoCM. Quadruplicate cultures were incubated in a humidified atmosphere of 5% CO_2 in air. Colonies were scored using a Leitz Diavert microscope. All values are expressed as mean ± 1 SD. When only CFU-GM were assayed, EPO and hemin were excluded from the cultures and replaced by an equal volume of IMDM, but all other culture conditions remained the same. CFU-GM derived colonies were scored at 3, 7, and 14 days. Total CFU-GM aggregates were defined as "colonies" containing two or more cells beginning at 3 days. In addition, at 7 and 14 days, the number of GM colonies >40 cells and >100 cells were enumerated.

To determine if the small, dispersed GM colonies or developing BFU-E in the IMDM negative controls were being stimulated by an (undetectable) accessory cell population, we cloned increasing numbers of cells (50, 100, 200, 500, 1,000, 2,000, and 4,000 cells/mL/35-mm dish). For detection of the GM colonies, no EPO was added to the cultures. Twelve cultures were prepared for each of the 3 lowest concentrations, 8 cultures were prepared for 500 cells/mL, and 4 cultures were prepared for each of the 3 highest concentrations. No increase was noted in either the incidence or size of the GM colonies at 7 or 14 days with increasing numbers of cells cloned. Similarly, in the BFU-E cultures, no increase was noted in the incidence or size of the BFU-E colonies at 14 days with increasing numbers of cells cloned.

To evaluate the morphological composition of colonies, individual colonies were aspirated into a finely drawn Pasteur pipette by capillary action and expressed onto a glass slide with a rubber bulb. The colony was immediately dried with a fine stream of compressed air and subsequently stained with May-Grunewald-Giemsa stain. The colonies were examined to determine whether they were neutrophilic, monocytic, and/or eosinophilic.

All the cell separation procedures, biological assays, and other experimental procedures described above used to examined the biological activities of the growth factors were conducted in the Laboratory of Hematopoietic Cell Kinetics in Sloan-Kettering Institute.

Purified Growth Factors

Purified biosynthetic (recombinant) GM-CSF and purified biosynthetic (recombinant) EPA were prepared as described.[1,2,5,12] and supplied by Dr David Golde and Dr Judith Gasson. Natural Ppo and Ppo α were prepared as reported[14,15] and supplied by Dr Karl Welte and Dr Janice Gabrilove. Recombinant Ppo, recently renamed granulocyte colony-stimulating factor (rhG-CSF), was kindly supplied by Dr Lawrence M. Souza, Amgen, Thousand Oaks, CA.[19] In this article, we have referred to recombinant Ppo as rPpo because this was the designation in use during the course of our experiments.

RESULTS

Purified Growth Factors

Purified biosynthetic (recombinant) GM-CSF was used in all studies. In preliminary experiments, GM-CSF was screened at concentrations from 5 to 1,000 pmol/L. At a concentration as low as 5 pmol/L, GM-CSF promoted the growth of CFU-GM and a subpopulation of BFU-E from enriched populations of human marrow progenitor cells. In most studies, GM-CSF was used at a concentration of ~100 pmol/L, but in a few studies 10 pmol/L was used and gave similar results. Increasing the concentration of GM-CSF to >100 pmol/L did not show any discernible increase in activity.

Purified biosynthetic (recombinant) EPA was used in all studies. EPA was screened at concentrations from 1 to 1,000 pmol/L. In our system, EPA had no significant activity on the growth of hematopoietic colonies from enriched populations of marrow progenitor cells at any concentration tested. After the initial screening, EPA was used at 10 pmol/L since higher concentrations have been reported to have an inhibitory effect.[2]

Both the natural Ppo purified from the 5637 cell line and biosynthetic (recombinant) Ppo (rPpo) were used in these studies at a concentration of 500 U/mL. In our initial studies, only the natural Ppo was available. In later experiments, only the recombinant Ppo (rPpo) was available; thus, we could not compare their activities on the same marrow specimens. The results using rPpo were within the range of values obtained with natural Ppo and therefore were not specifically designated in Figs 1, 2, 4, and 6. rPpo and Ppo were shown by others to have identical activity when compared on the same marrow population.[19]

The natural Ppo α purified from the 5637 human bladder carcinoma cell line was used in all studies at a concentration of 500 U/mL. This concentration showed similar activity to GM-CSF at a concentration of 10 and 100 pmol/L.

Ppo and Ppo α units were determined from dose-response curves and expressed as units per milliliter. Fifty units is the concentration stimulating a half-maximal colony number.[14]

Stimulation of Day 14 CFU-GM Derived Colonies From Enriched Populations of Normal Marrow Progenitor Cells

Using density separations and a panel of Mabs followed by immunoadherence (panning), normal bone marrow specimens were depleted of accessory and maturing cells to obtain enriched populations of progenitor cells by negative selection (a detailed description of procedures and recovery data is given in the Materials and Methods section). The activities of EPA, GM-CSF, Ppo α, and Ppo on the growth of day 14 CFU-GM from populations of enriched marrow progenitor cells are shown in Fig 1. The cloning efficiencies were normalized, and the MoCM positive control was taken as 100%. All GM colonies stimulated with MoCM were >40 cells, and most colonies had from several hundred to several thousand cells. EPA showed no significant activity over the IMDM-negative control in four experiments. CFU-GM

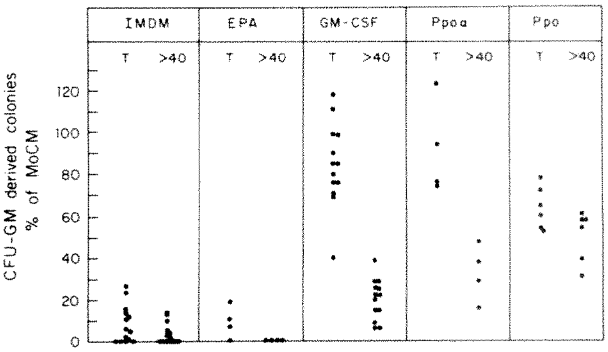

Fig 1. Total CFU-GM aggregates (T) and CFU-GM > 40 cells at 14 days are shown as a percentage of the MoCM control (conditioned medium from the Mo T cell line, normalized to 100%). The target populations were enriched marrow progenitor cells cloned at 1,500 to 5,000 cells/mL. The median cloning efficiency of day 14 CFU-GM derived colonies with MoCM was 1.2% (range 0.6% to 2.0%). Concentrations of the growth factors were: erythroid-potentiating activity (EPA) 10 pmol/L; GM-CSF 10 and 100 pmol/L; pluripoietin α (Ppo α) 500 U/mL; natural and recombinant pluripoietin (Ppo) 500 U/mL. In two studies rPpo was used, and in four studies natural Ppo was used. IMDM alone was the negative control. Four to eight 35-mm plates were counted for each value.

derived colonies seen in the IMDM negative controls and with EPA were small dispersed colonies at 14 days. With an increased concentration of methylcellulose (1.3%), it was easier to detect CFU-GM that were capable of developing without an exogenous source of stimulating factor(s). The number of colonies varied depending on the level of maturation of the target cell fraction. Some of these small colonies still existed at 7 and 14 days in 1.3% methylcellulose as opposed to cultures of more dilute methylcellulose (0.8% to 1.0%) in which they would have already largely dispersed. Only rarely did CFU-GM reach a size >40 cells in IMDM control cultures.

To determine if these small, dispersed GM colonies in the IMDM negative controls were being stimulated by an undetectable accessory cell population, we cloned increasing numbers of cells (range 50 to 4,000 cells/mL/35-mm dish) (data not shown a detailed description is given in the Materials and Methods section). No increase was noted at 7 or 14 days in either the incidence or size of these GM colonies with increasing numbers of cells cloned. All were dispersed colonies, and most were <20 cells. Because the number of GM colonies at 14 days was <1/50 cells, the data suggest that these CFU-GM were capable of limited divisions in the absence of mediation by an accessory cell population. Whether this latter limited proliferation is an intrinsic charcteristic of this small population of CFU-GM or is due to unknown factors present in FCS is unknown.

Ppo α and GM-CSF had very similar activities when compared on four marrow populations, both in the numbers of total CFU-GM aggregates and CFU-GM >40 cells. Taken together, Ppo α (4 marrows) and GM-CSF (13 marrows) gave a mean of 86% (±20) (total CFU-GM aggregates) and 23% (±12) (CFU-GM >40 cells) of those obtained with MoCM at 14 days. Morphological examination of day 14 CFU-GM derived colonies stimulated by GM-CSF revealed that most of the small colonies were neutrophilic and monocytic whereas most of the larger colonies were eosinophilic. On the other hand, day 14 CFU-GM derived colonies stimulated by MoCM were almost all large, and morphological examination showed them to be neutrophilic, monocytic, and eosinophilic. The ratio of morphological cell types varied with the donor.

The activity of Ppo showed less variance between the total number of day 14 CFU-GM aggregates [mean 64% (±10)] and CFU-GM >40 cells [mean 50% (±12)] of MoCM on six marrow populations. When examined morphologically, day 14 CFU-GM derived colonies stimulated by Ppo were neutrophilic and monocytic; no eosinophilic colonies were seen. It should be emphasized that even the larger CFU-GM derived colonies stimulated by GM-CSF, Ppo α, and Ppo were almost invariably much smaller than CFU-GM derived colonies stimulated by MoCM.

Stimulation of Day 14 CFU-GM Derived Colonies From Enriched Populations of Normal Marrow Progenitor Cells Following Freezing

Even though as little as 0.5% of buffy coat myelocytes is recovered following immunoadherence, myelocytes are still the major contaminating cell population after panning. To enrich marrow progenitor cells further, we froze postpanning marrow populations since freezing destroys more mature progenitors, including myelocytes. The mean percentage of blasts and promyelocytes after freezing was 77% (53% to 100%) and 14% (0% to 34%), respectively. The mean recovery of day 14 CFU-GM was 39% (10% to 71%), and the CFU-GM that survived freezing formed large healthy colonies with MoCM (all were >40 cells, and most contained several hundred to several thousand cells).

In Fig 2, the activities of GM-CSF, Ppo α, and Ppo on the growth of day 14 CFU-GM derived colonies from enriched populations of marrow progenitor cells following freezing are shown. The ability of GM-CSF and Ppo α to stimulate day 14 CFU-GM after freezing was markedly reduced. The largest reduction was in total CFU-GM aggregates (mean 86% of MoCM prior to freezing (Fig 1) as compared with a mean of 19% (±7) of MoCM on postfreezing populations) and a somewhat smaller reduction in their ability to stimulate CFU-GM >40 cells (mean 23% of MoCM prior to freezing as compared with a mean of 10% (±6) of MoCM on postfreezing populations). Freezing, therefore, apparently destroyed most of the CFU-GM that gave rise to small colonies (<40 cells) seen in day 14 cultures stimulated by GM-CSF. Day 14 CFU-GM derived colonies from one postfreezing marrow population, stimulated by GM-CSF, were eosinophilic, monocytic (and rarely macrophage) colonies. No small neutrophilic colonies were seen with GM-CSF, as was the case in marrow populations prior to freezing. From the same marrow, day 14 CFU-GM derived colonies stimulated by MoCM were eosinophilic, promonocytic, and neutrophilic.

As shown in Fig 2, the degree of reduction in the ability of Ppo to stimulate day 14 CFU-GM in postfreezing marrow populations was more variable; in three experiments there was a striking reduction in the activity of Ppo, whereas in two other experiments the reduction was less pronounced. On morphological examination, day 14 CFU-GM derived colo-

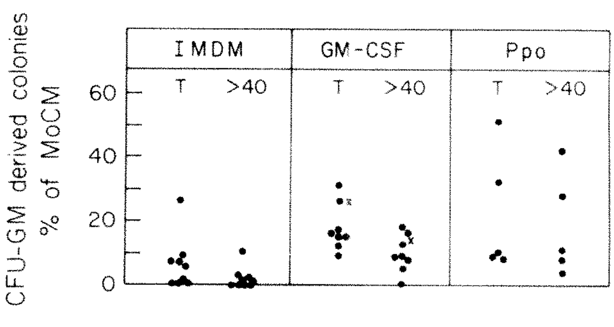

Fig 2. Total CFU-GM aggregates (T) and CFU-GM > 40 cells at 14 days are shown as a percentage of the MoCM control (conditioned medium from the Mo T cell line, normalized to 100%). The target populations were enriched marrow progenitor cells following freezing cloned at 400 to 1,200 cells/mL. The median cloning efficiency of day 14 CFU-GM derived colonies with MoCM was 3.2% (range 1.3% to 7.0%). Concentrations of the growth factors were: GM-CSF 10 and 100 pmol/L; pluripoietin α (Ppo α) (X) in one study 500 U/mL; natural and recombinant pluripoietin (Ppo) 500 U/mL. In two studies rPpo was used and in three studies natural Ppo was used. IMDM alone was the negative control. Four to eight 35-mm plates were counted for each value.

Table 1. Growth of CFU-GM–Derived Colonies From Enriched Marrow Progenitor Cell Populations Before and After Freezing

	3 Days		7 Days				14 Days					
	Total		Total		>40 Cells		Total		>40 Cells		>100 Cells	
	CE (%)	*	CE (%)	*	CE (%)	*	CE (%)	*	CE (%)	*	CE (%)	*
A†												
MoCM	3.7		3.4		0.6		1.3		1.3		0.9	
IMDM	0.5	(14)	0.8	(24)	0	(0)	0	(0)	0	(0)	0	(0)
GM-CSF	2.0	(54)	2.0	(59)	0	(0)	0.9	(69)	0.2	(15)	0.03	(3)
Ppo	1.7	(46)	2.1	(62)	0.2	(33)	0.7	(54)	0.5	(39)	0.03	(3)
B‡												
MoCM	4.1		4.7		1.3		2.5		2.5		2.3	
IMDM	0	(0)	0.2	(4)	0	(0)	0	(0)	0	(0)	0	(0)
GM-CSF	0.2	(5)	0.4	(9)	0	(0)	0.4	(16)	0.4	(16)	0.2	(9)
Ppo	0.5	(12)	0.5	(11)	0	(0)	0.8	(32)	0.7	(28)	0.1	(4)

CE, cloning efficiency; MoCM, conditioned medium from Mo T cell line; IMDM, Iscove's modified Dulbecco's medium; Ppo, natural and recombinant pluripoietin.

*Percent of MoCM.

†Population of enriched marrow progenitor cells before freezing (blasts 30%; promyelocytes 15%; myelocytes 42%) cloned at 2,400 cells/mL.

‡Population of enriched marrow progenitor cells postfreezing (blasts 73%; promyelocytes 23%; myelocytes 0%) cloned at 1,200 cells/mL. Concentration of GM-CSF was 100 pmol/L; Ppo concentration was 500 U/mL. For population A, rPpo was used; for population B, natural Ppo was used.

nies from postfreezing marrow populations, stimulated by Ppo, were mature neutrophilic, monocytic (and rarely macrophage) colonies.

The larger CFU-GM derived colonies stimulated by Ppo, GM-CSF, and Ppo α were almost always much smaller than CFU-GM derived colonies stimulated by MoCM.

Comparison of Activities of GM-CSF and Ppo on Growth of CFU-GM Derived Colonies at 3, 7, and 14 Days

The development of CFU-GM derived colonies from enriched marrow progenitor cell populations was followed by serially evaluating the number and size of CFU-GM derived colonies at 3, 7, and 14 days. Table 1 shows the results of a representative experiment before and after freezing. GM-CSF stimulated a large number of small CFU-GM derived colonies (<40 cells) from the unfrozen marrow population at 7 days, and at 14 days in these same cultures, the majority of the GM colonies present (~80%) were still <40 cells. Thus, only a small number of the CFU-GM derived colonies reached a size >40 cells, and only a rare CFU-GM derived colony obtained a size >100 cells as compared with CFU-GM derived colonies stimulated by MoCM. In contrast, after freezing, the population of small CFU-GM stimulated by GM-CSF was not present in the marrow population, and only larger colonies survived. Although all the CFU-GM stimulated by GM-CSF at 7 days were <40 cells, at 14 days all were >40 cells and 50% of these were >100 cells. The activity of GM-CSF on CFU-GM >40 cells, when compared with the activity of MoCM, was similar in both marrow populations (before and after freezing) at 14 days (>40 cells 15% to 16% and >100 cells 3% to 9% of MoCM). The data indicate that the reduction in activity of GM-CSF on growth of CFU-GM derived colonies in marrow populations following freezing is therefore essentially due to a loss of CFU-GM with limited proliferative capacity in postfreezing marrow populations.

The reduction in activity of Ppo on growth of CFU-GM derived colonies in postfreezing marrow populations was greatest at 7 days (total CFU-GM aggregates 62% of MoCM prior to freezing as compared with 11% of MoCM after freezing, and CFU-GM derived colonies >40 cells 33% of MoCM prior to freezing as compared with 0% of MoCM after freezing). At 14 days, the activity of Ppo when compared with MoCM was less reduced following freezing (the major reduction being in CFU-GM derived colonies <40 cells). Therefore, these results indicate that the reduction in activity of Ppo on growth of CFU-GM in marrow populations following freezing is primarily due to a loss of a subpopulation of day 7 CFU-GM derived colonies.

Velocity Sedimentation of Enriched Marrow Progenitor Cell Populations in a Linear Ficoll Isokinetic Gradient

To characterize the nature of the progenitors stimulated by GM-CSF further, populations of enriched normal marrow progenitor cells were separated on a Ficoll isokinetic gradient to separate the progenitors on the basis of cell size. Based on previous experience with this gradient, we obtained

Table 2. Differentials of the Three Pooled Fractions From the Linear Ficoll Gradient and the Distribution of Individual Cell Types in the Gradient

	Differential (%)					Distribution in the Gradient Percentage of Total			
Fraction	Bl	Pro	Myel	Ly	Other	Bl	Pro	Myel	Ly
F4-10	70	9	5	13	3	76	16	3	93
F11-12	23	22	50	1	4	18	25	22	7
F13-15	4	22	73	0	1	6	59	75	0

A population of enriched marrow progenitor cells following panning was separated on a linear Ficoll isokinetic gradient. Left: differentials are shown for the three pooled fractions: Bl, blasts; Pro, promyelocytes; Myel, myelocytes; Ly, lymphocytes. Right: distribution for each cell type in the gradient. The latter was calculated by normalizing the total of each cell type recovered in the gradient to 100. Same marrow gradient as that shown in Figs 3 and 7 and Tables 3 and 4.

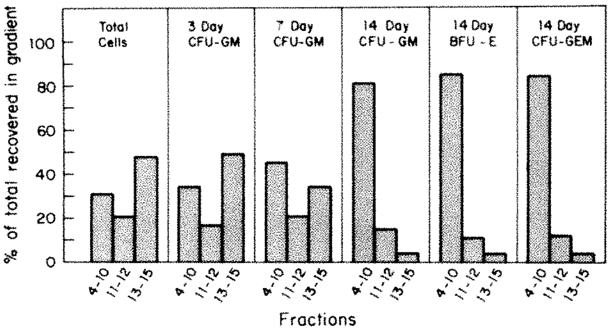

Fig 3. The total number of nucleated cells and the total number of CFU-GM, BFU-E, and CFU-GEM (stimulated by MoCM) recovered in the linear Ficoll isokinetic gradient were normalized to 100, and their distribution in the pooled fractions is shown as a percentage of the total. Same marrow gradient as that shown in Tables 2, 3, and 4, and Fig 7.

three pooled fractions. F4-10 is the small cell fraction, F11-12 contains cells intermediate in size, and F13-15 is the large cell fraction. Similar results were obtained in four gradients and we illustrate the results with one representative study.

Of the cells layered on the gradient, we recovered 51% of all nucleated cells in the three fractions: 100% of the blasts, 60% of the promyelocytes, 40% of the myelocytes, and 24% of the lymphocytes. The cloning efficiencies of the unfractionated panned population were: CFU-GM 1.5%, BFU-E 2.4%, and CFU-GEM 0.02%. The cloning efficiencies of the total panned population recovered from the gradient (51% of the total cells layered) were: CFU-GM 3.3%, BFU-E 4.0%, and CFU-GEM 0.08%. Therefore, 100% of the absolute number of colonies derived from CFU-GM, BFU-E, and CFU-GEM layered on the gradient were recovered in the three fractions.

Table 2 shows the differentials of each fraction and the distribution of each cell type in the gradient. F4-10 contained 70% blasts (and these represented 76% of the total blasts in the gradient) in contrast to only 4% blasts in F13-15. The mature cell types increased with increasing cell size as evidenced by F13-15, which contained 73% myelocytes. (The myelocytes recovered in the gradient represent only 0.2% to 1.5% of buffy coat myelocytes). Essentially all lymphocytes were in fraction 4-10, and they represent <0.1% of buffy coat lymphocytes following panning.

Figure 3 shows the distribution of total cells and the distribution of hematopoietic colonies stimulated by MoCM in the three fractions. The distribution of CFU-GM is shown at 3, 7, and 14 days. Whereas only 34% of CFU-GM aggregates were in F4-10 at 3 days, 81% of day 14 CFU-GM stimulated by MoCM were in F4-10; 84% to 85% of day 14 BFU-E and CFU-GEM stimulated by MoCM were also in the small cell fraction (F4-10).

Table 3 shows the cloning efficiencies of CFU-GM stimulated by MoCM and GM-CSF at 3, 7, and 14 days for the total postpanning marrow population and the three fractions obtained from the Ficoll isokinetic gradient.

GM-CSF showed increasing activity on growth of total CFU-GM aggregates with increasing size of the progenitor cells. In F13-15, GM-CSF gave a slightly increased number

Table 3. Cloning Efficiencies of CFU-GM Derived Colonies in the Total Postpanning Population and the Three Fractions From the Linear Ficoll Gradient

	3 Days CE (%)	7 Days CE (%)		14 Days CE (%)		
	Total	Total	>40 Cells	Total	>40 Cells	>100 Cells
Total Panned Population*						
MoCM	8.1	8.9	3.6	3.3	3.3	3.1
GM-CSF	5.4	7.6	0.1	2.9	0.8	0.2
(% of MoCM)	(67)	(85)	(3)	(88)	(24)	(6)
F4-10						
MoCM	8.9	12.8	4.7	8.6	8.6	8.1
GM-CSF	2.0	5.6	0.3	3.3	1.6	0.5
(% of MoCM)	(22)	(44)	(6)	(38)	(19)	(6)
F11-12						
MoCM	6.5	9.0	3.0	2.3	2.3	2.2
GM-CSF	4.2	7.9	0.05	4.4	0.6	0.05
(% of MoCM)	(65)	(88)	(2)	(191)	(26)	(2)
F13-15						
MoCM	8.4	6.3	3.2	0.3	0.3	0.3
GM-CSF	8.2	8.8	0.06	1.9	0.4	0.05
(% of MoCM)	(98)	(140)	(2)	(633)	(133)	(17)

Same marrow gradient as that shown in Tables 2 and 4 and Figs 3 and 7. The cloning efficiencies (CE %) of CFU-GM stimulated by MoCM and GM-CSF are shown at 3, 7, and 14 days for the total postpanning marrow population and the three fractions obtained following separation of the postpanning marrow cells on a linear Ficoll isokinetic gradient. Number of cells cloned for each population were: total postpanning population 3,400 cells/mL; fraction 4-10 1,200 cells/mL; fraction 11-12 2,000 cells/mL; fraction 13-15 5,000 cells/mL. The concentration of GM-CSF was 100 pmol/L.

*Total panned population recovered from the gradient. This represented 51% of the total cells in the unfractionated panned population. Because the cloning efficiency of CFU-GM in the unfractionated population was 1.5% with MoCM at 14 days and more than double (3.3%) in the population recovered from the gradient, 100% of the CFU-GM was recovered.

Abbreviations as in Table 1.

of total CFU-GM aggregates at 7 days, as compared with MoCM; at 14 days, there were six times (633%) as many CFU-GM aggregates with GM-CSF as compared with MoCM. Of these latter GM colonies, 79% were <40 cells, however. To a lesser degree, there was an increased number of F11-12 total CFU-GM aggregates at 14 days in GM-CSF stimulated cultures as compared with MoCM. Of these latter GM colonies, 86% were <40 cells, however. Of the total cells recovered from the gradient, 69% were in F11-15 (Fig 3). Therefore, the increased cloning efficiency of small GM colonies (<40 cells) in F11-12 and F13-15 primarily accounted for the high total number of GM colonies recovered from the gradient at 14 days in GM-CSF stimulated cultures (88% of the GM colonies recovered from the gradient with MoCM). As shown in Fig 3, the distribution of the total day 14 GM colonies in MoCM stimulated cultures was: F4-10 81%, F11-12 15%, and F13-15 4%. In contrast, the distribution of day 14 GM colonies in GM-CSF stimulated cultures was: F4-10 35%, F11-12 33%, and F13-15 32% (not shown). Therefore, at 14 days, only 19% of the GM colonies in MoCM stimulated cultures were derived from progenitor cells in F11-15, in contrast to 65% of the GM colonies in GM-CSF stimulated cultures. This would explain why 94% of the total day 14 GM colonies in MoCM stimulated cultures were >100 cells, whereas GM-CSF stimulated cultures contained only 6% of the number obtained with MoCM.

The most probable reason for the increased number of small GM colonies (<40 cells) derived from the larger CFU-GM progenitors in F11-15 in day 14 GM-CSF stimulated cultures is that GM-CSF, which is known to inhibit neutrophil migration, prevented these small, mature GM colonies from dispersing. The peak of F13-15 CFU-GM derived colonies >40 cells stimulated by MoCM was at 7 days and most of these mature GM colonies had dispersed by 14 days. In contrast, the peak of CFU-GM derived colonies >40 cells in GM-CSF stimulated cultures from this same fraction was at 14 days. The peak with MoCM was 3.2%, however, and was only 0.4% with GM-CSF.

GM-CSF showed the least relative activity on growth of day 14 CFU-GM derived colonies from the small cell fraction (F4-10) (38% of the number stimulated by MoCM). In addition, at 14 days, all the GM colonies stimulated by MoCM from this latter fraction were >40 cells and 94% of them were >100 cells. In contrast, only 48% of the GM colonies stimulated by GM-CSF were >40 cells and only 15% of them were >100 cells.

To determine if these small GM colonies in GM-CSF stimulated cultures were derived from contaminating mature CFU-GM, we assayed F4-10 both before and after freezing; as previously noted, freezing selectively destroyed the more mature progenitors, especially myelocytes. Before freezing, the differential of myeloid precursors in F4-10 was blasts 70%, promyelocytes 9%, and myelocytes 5% (Table 2), whereas after freezing it was blasts 86%, promyelocytes 11%, and myelocytes 0%. With MoCM stimulation, the total cloning efficiency of CFU-GM dropped only from 8.6% to 7.0% following freezing. In contrast, with GM-CSF stimulation, the total cloning efficiency of CFU-GM fell from 3.3% to 0.8% (a 76% reduction *v* a 19% reduction with MoCM). The activity of GM-CSF on day 14 F4-10 CFU-GM, as a percentage of MoCM, before and after freezing was 38% *v* 12% (total CFU-GM aggregates), 19% *v* 9% (CFU-GM > 40 cells), and 6% *v* 4% (CFU-GM > 100 cells). Thus, the greatest reduction was in the smaller GM aggregates, strongly supporting the argument that most day 14 CFU-GM stimulated by GM-CSF arose from the contaminating mature progenitors.

Morphological examination of colonies derived from enriched progenitors not subjected to freezing at 7 days showed that both MoCM and GM-CSF promoted the growth of neutrophilic and monocytic colonies. MoCM colonies, however, were much larger. At 14 days, GM-CSF cultures had many small mature neutrophilic colonies, some small monocytic colonies, and some larger eosinophilic colonies; the latter accounted for most of the larger colonies. At 14 days, MoCM colonies were neutrophilic, eosinophilic, and monocytic.

Stimulation of Day 14 CFU-GM Derived Colonies From Enriched Populations of Normal Blood Progenitor Cells

Using density separations and a panel of monoclonal antibodies followed by immune rosetting, we depleted normal peripheral blood specimens of accessory and maturing cells to obtain enriched populations of progenitor cells by negative selection (described in the Materials and Methods section).

Figure 4 shows the activity of GM-CSF, Ppo α, and Ppo on the growth of day 14 CFU-GM derived colonies from enriched populations of peripheral blood progenitor cells. The cloning efficiencies were normalized, and the MoCM positive control was taken as 100%.

GM-CSF and Ppo α activities were similar when compared on three blood populations. Taken together, GM-CSF and Ppo α gave a mean of 48% (±13) (total CFU-GM aggregates) and 29% (±13) (CFU-GM derived colonies > 40 cells) of those obtained with MoCM at 14 days. On

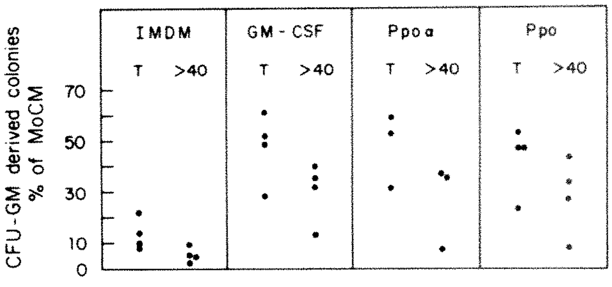

Fig 4. Total CFU-GM aggregates (T) and CFU-GM > 40 cells at 14 days are shown as a percentage of the MoCM control (conditioned medium from the Mo T cell line, normalized to 100%). The target populations were enriched populations of peripheral blood progenitor cells; 600 to 2,000 cells/mL were cloned. The median cloning efficiency of the MoCM control was 1.3% (range 0.6% to 3.2%). The concentrations of the growth factors were: GM-CSF 100 pmol/L; pluripoietin α (Ppo α) 500 U/mL; natural or recombinant pluripoietin (Ppo) 500 U/mL. Natural Ppo was used in the four studies. IMDM alone was the negative control. Eight 35-mm plates were counted for each value.

morphological examination, day 14 blood CFU-GM stimulated by GM-CSF and Ppo α were eosinophilic and monocytic whereas those stimulated by MoCM were neutrophilic in addition to eosinophilic and monocytic. Even though most of the eosinophilic colonies stimulated by GM-CSF and Ppo α were >40 cells, they were much smaller than eosinophilic colonies stimulated by MoCM. The monocytic colonies from the blood stimulated by GM-CSF and Ppo α were generally very small, whereas those stimulated by MoCM contained several hundred cells.

Ppo stimulated a mean of 43% (±13) (total CFU-GM aggregates) and 28% (±15) (CFU-GM derived colonies >40 cells) of the day 14 blood CFU-GM obtained with MoCM. When examined morphologically, day 14 blood CFU-GM derived colonies stimulated by Ppo were neutrophilic and monocytic (no eosinophilic colonies were seen). The ratio of morphological cell types varied with the donor. Even the larger CFU-GM derived colonies stimulated by Ppo were much smaller than CFU-GM stimulated by MoCM.

Effect of Delayed Addition of EPO on Growth of BFU-E

Before evaluating the activities of the purified growth factors on growth of BFU-E derived colonies, we performed preliminary experiments to study the effects of delaying the addition of EPO to the cultures. The purpose was to reduce the population of BFU-E, immediately dependent on EPO for proliferation and terminal differentiation but not requiring an exogenous source of BPA (ie, the most mature BFU-E). Figure 5 shows the results of delaying the addition of EPO for 24 and 48 hours. By delaying EPO addition for 48 hours, the number of BFU-E derived colonies in the background control (IMDM alone) for marrow was reduced from a mean of 56% (±12) of MoCM (EPO added initially) to a mean of 4% (±2) of MoCM (EPO added at 48 hours). In blood, the IMDM control was reduced from a mean of 19% (±6) of MoCM (EPO initially) to a mean of 5% (±4) of MoCM (EPO at 48 hours).

Figure 5 also shows that a population of BFU-E (probably the most mature BFU-E) did not survive, even in the initial presence of MoCM, when EPO was delayed for 48 hours [a mean of 16% (±9) of the BFU-E in marrow and 26% (±15) in the blood]. In addition, the data suggest that the population of BFU-E responding to EPO at 48 hours is composed of erythroid progenitors that require a factor or factors in MoCM to respond to EPO (those above background with EPO added initially) and a population of erythroid progenitors that require a factor or factors in MoCM to survive if EPO addition is delayed (a portion of those BFU-E that could respond to EPO initially in the IMDM control).

Effects of Purified Growth Factors on the Growth of BFU-E Derived Colonies From Enriched Populations of Normal Marrow Progenitor Cells in the Initial Presence of EPO and After Delaying Addition for 48 Hours

When EPO was added initially to the cultures, consistent significant activities of the purified growth factors on the growth of BFU-E from prefreezing marrow populations could not be clearly demonstrated (data not shown). EPA and Ppo gave no significant increase in either the size or the number of BFU-E over that seen in the IMDM (negative) control. Ppo α gave 75% of the number of BFU-E in the IMDM control cultures on one marrow population and 15% above the number in the IMDM cultures on another marrow population. GM-CSF, when compared with the number of BFU-E in the IMDM cultures, gave the same number on 4 marrows, 75% to 80% of the number on 2 marrows, 15% to 20% above the number on 5 marrows, and 35% to 55% above the number in the IMDM control cultures on 2 marrows. The results were similar on target populations postfreezing with EPO added initially.

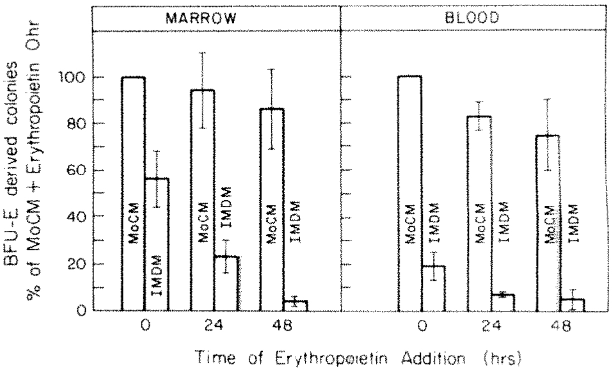

Fig 5. Effects of delayed addition of erythropoietin (EPO) on the growth of BFU-E derived colonies is shown as a percentage of the MoCM control (conditioned medium from the Mo T cell line, normalized to 100% with EPO added initially). The results are mean values of 12 marrow and six blood populations ± 1 SD. IMDM was the negative control. The number of cells cloned for blood was 600 to 2,000 cells/mL; for marrow, it was 2,000 to 6,000 cells/mL. The median cloning efficiency for the blood populations was 4.4% (range 0.9% to 9.7%); for the marrow populations, it was 1.2% (range 0.6% to 1.7%). Quadruplicate 35-mm plates were counted for each value.

The activities of GM-CSF, Ppo α, Ppo, and EPA on the growth of BFU-E derived colonies from enriched marrow progenitor cell populations (before and after freezing), could best be demonstrated by delaying addition of EPO for 48 hours to reduce background (ie, BFU-E that can respond to EPO initially without an exogenous source of BPA). The data are shown in Fig 6. EPA showed no significant activity over the IMDM control on the growth of BFU-E prior to freezing. Ppo also showed no significant activity over the IMDM control on the growth of BFU-E either before or after freezing.

The activities of GM-CSF and Ppo α on the growth of BFU-E derived colonies were similar when compared before freezing. Taken together, GM-CSF (12 marrows) and Ppo α (4 marrows) gave a mean of 37% (±14) of the BFU-E derived colonies obtained with MoCM.

The presence or absence of hemin in the cultures did not appear to alter the level of the activity of any of the factors on prefreezing marrow populations. Hemin added to the cultures generally improves the size and hemoglobinization of BFU-E derived colonies, although in cultures stimulated with MoCM addition of hemin does not significantly effect the total number of BFU-E derived colonies. (The number of BFU-E without hemin is generally ~85% of the number with hemin.) GM-CSF and Ppo α (single marrow) showed a

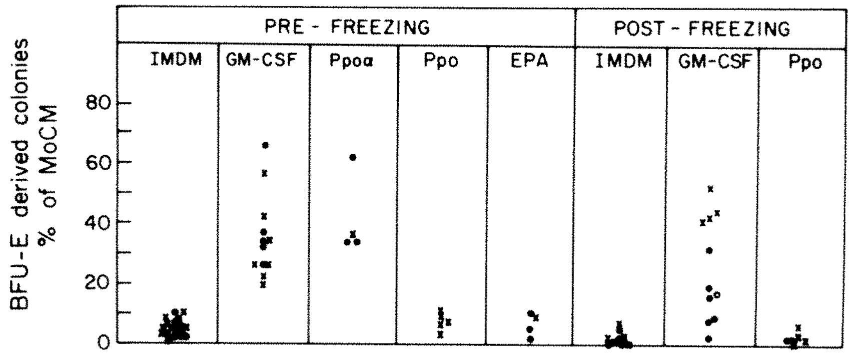

Fig 6. BFU-E derived colonies are shown as a percentage of the MoCM control (conditioned medium from the Mo T cell line, normalized to 100% with erythropoietin (EPO) added at 48 hours). The target populations were enriched marrow progenitor cells before and after freezing. For marrow populations before freezing, 1,500 to 5,000 cells/mL were cloned; for marrow populations after freezing, 400 to 1,200 cells/mL were cloned. The median cloning efficiency of BFU-E derived colonies with MoCM and EPO addition at 48 hours for prefreezing marrow populations was 1.0% (0.5% to 2.5%) and for postfreezing marrow populations was 4.3% (1.8% to 12.0%). Concentrations of the growth factors were: GM-CSF 10 and 100 pmol/L; pluripoietin α (Ppo α) 500 U/mL; natural and recombinant pluripoietin (Ppo) 500 U/mL; erythroid-potentiating activity (EPA) 10 pmol/L. In two prefreezing and three postfreezing studies rPpo was used, and in three prefreezing and three postfreezing studies, natural Ppo was used. Cultures without hemin (●); cultures with hemin (X). Ppo α activity on one postfreezing marrow population (O). Quadruplicate 35-mm plates were counted for each value.

reduction in activity on the growth of BFU-E derived colonies from postfreezing marrow populations when hemin was absent from the cultures. Taken together, GM-CSF and Ppo α gave a mean of 15% (±10) of the number of BFU-E derived colonies obtained with MoCM in the absence of hemin. When hemin was added to the cultures of postfreezing marrow populations, however, the activity of GM-CSF increased and was now similar to the activity of GM-CSF on prefreezing marrow populations. GM-CSF gave a mean of 45% (±5) of the number of BFU-E derived colonies obtained with MoCM in the presence of hemin. We are currently investigating the significance of this observation.

Effects of Purified Growth Factors on the Growth of BFU-E in Marrow Fractions From the Linear Ficoll Gradient

The growth of BFU-E derived colonies in the three fractions from the isokinetic gradient was determined with MoCM, with IMDM alone, and with GM-CSF. The results are shown in Fig 7 as a percentage of MoCM with EPO added initially and with the addition of EPO delayed for 48 hours. With EPO added initially, the "background" BFU-E derived colonies in the IMDM cultures increased with increasing size of the progenitors (F4-10 50%, F11-12 78%, and F13-15 94% of the number with MoCM). GM-CSF showed little or no activity over the IMDM control when EPO was added initially. When EPO was delayed for 48 hours, the number of BFU-E with MoCM was ~80% of the number of BFU-E with EPO added initially in all three fractions (data not shown). The number of background BFU-E in the IMDM cultures was reduced to ~11% of the BFU-E with MoCM.

The activity of GM-CSF on the growth of BFU-E could be clearly demonstrated when the addition of EPO was delayed for 48 hours. GM-CSF showed slightly more activity on the growth of BFU-E derived colonies in F11-12 and F13-15 than on the growth of BFU-E in F4-10.

Effects of Purified Growth Factors on the Growth of BFU-E From Enriched Populations of Normal Blood Progenitor Cells

Ppo showed no significant activity (over the IMDM negative control), on the growth of BFU-E derived colonies from four enriched populations of normal blood progenitor cells. The results were the same whether EPO was added initially or at 48 hours (data not shown).

GM-CSF (four blood populations) and Ppo α (three blood populations) had similar activity (data not shown). When EPO was added initially, both factors consistently showed significant activity, but the degree of activity varied greatly (1.3 to 3.7 times the number of BFU-E in the IMDM negative control). This result is in contrast to EPO added initially to marrow progenitor cells, in which there was no

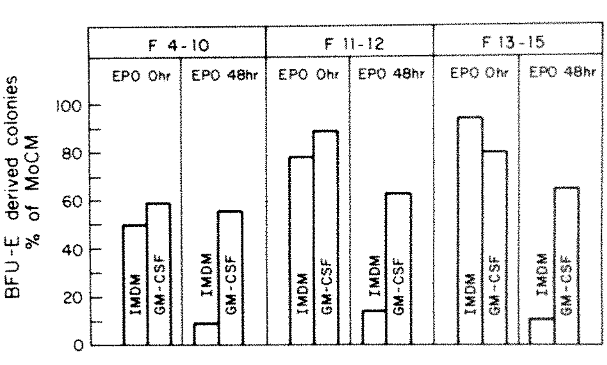

Fig 7. Same marrow gradient as that shown in Tables 2, 3, and 4 and Fig 3. Results are shown as a percentage of the number of BFU-E derived colonies obtained with conditioned medium from the Mo T cell line (MoCM). The number of cells cloned for each fraction were F4-10 1,200 cells/mL, F11-12 2,000 cells/mL, and F13-15 5,000 cells/mL. The concentration of GM-CSF was 100 pmol/L. The cloning efficiency of BFU-E stimulated by MoCM with erythropoietin (EPO) added initially for each fraction was F4-10 14.2%, F11-12 2.6%, and F13-15 0.4%. Quadruplicate 35-mm plates were counted for each value.

consistent increase over the IMDM control with GM-CSF and Ppo α. When the addition of EPO was delayed for 48 hours, GM-CSF and Ppo α together gave a mean of 47% ($\pm$17) of the BFU-E derived colonies obtained with MoCM. Their activities on the growth of BFU-E from blood progenitor populations were therefore similar to their activities on growth of marrow BFU-E derived colonies when EPO addition was delayed for 48 hours (a mean of 37% of MoCM for marrow progenitors).

Our results show that GM-CSF and Ppo α affect the growth of only a subpopulation of BFU-E in both marrow and blood when compared with MoCM. With EPO addition at 48 hours, the size of the subpopulation of BFU-E whose growth was affected by GM-CSF and Ppo α was similar in both marrow and blood (between 20% and 45% of the total BFU-E population stimulated with MoCM). With EPO added initially, there was more variability. A significant increase over the IMDM cultures occurred in 2 of 13 marrows and 4 of 4 blood populations, but only a slight increase was seen in 5 of 13 marrows and no increase was seen in 6 of 13 marrows. Clearly, marrow BFU-E populations generally contain a significantly larger subpopulation of mature BFU-E that can respond to EPO initially without an exogenous source of growth factor(s) (IMDM cultures, Fig 5) than do blood BFU-E populations. Our data also suggest that a large portion of this latter population can survive with MoCM, GM-CSF, and Ppo α when EPO addition is delayed for 48 hours, but does not survive without these factor(s). In addition, our data suggest that there is an additional subpopulation of BFU-E derived colonies (more consistently seen in blood) whose growth may be affected by GM-CSF and Ppo α when EPO is added initially. This second subpopulation may be at an intermediate stage of maturation but this has not been definitively shown. Further studies are necessary to define the stages of maturation of the subpopulations of BFU-E in both marrow and blood.

Effects of Purified Growth Factors on the Growth of CFU-GEM Derived Colonies From Enriched Populations of Marrow and Blood Progenitor Cells

EPA (4 marrows) and Ppo (11 marrows and 4 bloods) showed no detectable activity on the growth of CFU-GEM derived colonies from marrow or blood either before or after freezing.

Considering only those marrow and blood populations from which CFU-GEM derived colonies were obtained with MoCM, GM-CSF stimulated the growth of CFU-GEM from 10 of 18 marrow and 2 of 4 blood populations. The CFU-GEM derived colonies obtained with GM-CSF were almost always much fewer and smaller in size than CFU-GEM derived colonies obtained with MoCM. Ppo α gave similar activity when compared with GM-CSF on four marrow and three blood populations. The addition of hemin significantly enhanced the ability of GM-CSF and Ppo α to promote the growth of CFU-GEM derived colonies.

Table 4 shows the activity of MoCM and GM-CSF on the growth of CFU-GEM derived colonies from three fractions of the linear Ficoll gradient (same gradient as shown in Tables 2 and 3 and Figs 3 and 7). The total absolute number of CFU-GEM derived colonies stimulated by MoCM recovered from the gradient was normalized to 100. GM-CSF promoted the growth of 18% of the CFU-GEM derived colonies obtained with MoCM in the total panned population. When the same marrow population was separated in the linear Ficoll gradient (the distribution in the gradient of the CFU-GEM derived colonies obtained with MoCM is shown in Fig 3), GM-CSF promoted the growth of 15% of the CFU-GEM obtained with MoCM in F4-10, 26% of the number with MoCM in F11-12, and 71% of the number with MoCM in F13-15.

DISCUSSION

To examine the activities of EPA, GM-CSF, Ppo α, and Ppo, we prepared enriched populations of human marrow and blood progenitor cells. The target populations were therefore depleted of accessory cells, and the progenitor cells were concentrated so that relatively few cells were required for assay. We used a relatively high concentration of methylcellulose to reduce the migration of cells and examined the direct activities of the purified factors by cloning at cell numbers low enough to give relatively few colonies, thus avoiding possible indirect stimulation by developing colonies. The relative activities of the growth factors were determined on progenitor cell populations from several donors, and the results were found to be consistent.

Because some growth of small, dispersed GM colonies in the IMDM negative controls was observed, we cloned increasing numbers of cells in the IMDM cultures to determine if an undetectable (by FACS analysis) accessory cell population was stimulating these progenitor cells. As few as 50 cells/mL/35-mm dish gave the same incidence and size of these colonies as did the highest number tested, 4,000 cells/mL/35-mm dish. Since the incidence was <1 GM colony/50 cells, the data strongly suggest that these progenitors were capable of limited divisions without stimulation by accessory cells and that our results indicate that the activities of the growth factors were direct effects. That some CFU-

Table 4. Number of CFU-GEM Derived Colonies/10^6 Cells in the Total Postpanning Population and the Three Fractions From the Linear Ficoll Gradient

	Total Panned Population	Percent of MoCM	Fraction 4-10	Percent of MoCM	Fraction 11-12	Percent of MoCM	Fraction 13-15	Percent of MoCM
MoCM	82		220		47		7	
GM-CSF	15	(18)	33	(15)	12	(26)	5	(71)

Same marrow gradient as that shown in Tables 2 and 3 and Figs 3 and 7. The number of cells cloned for each fraction were: F4-10 1,200 cells/mL; F11-12 2,000 cells/mL; and F13-15 5,000 cells/mL. The concentration of GM-CSF was 100 pmol/L. Eight 35-mm culture plates were counted for each value.

GM are capable of limited proliferation in the absence of any contribution by non–colony-forming cells may be due to factors present in the FCS or CSF–receptor complexes existing prior to ex vivo removal of the cells from the donors.[69] In more dilute semisolid culture conditions (0.3% agar or 0.8% to 1.0% methylcellulose), these dispersed colonies would go undetected due to complete dispersion except at times early after the initiation of culture.

Before evaluating the activities of the factors on the growth of BFU-E derived colonies, we examined the effect of delaying the addition of EPO to reduce the number of BFU-E that could respond to EPO without requiring the addition of an exogenous source of BPA. Several investigators have previously delayed the addition of EPO to study the effects of crude sources of BPA and to distinguish subpopulations of BFU-E[70-75]; more recently, we and others have done so to examine the activities of purified growth factors.[6-10,76,77] In our studies, delaying the addition of EPO for 48 hours reduced the number of BFU-E derived colonies in the background control cultures (IMDM alone) to a mean of 4% to 5% of the number in cultures with MoCM (see Fig 5). In addition, there was a population of BFU-E, presumably the most mature BFU-E, that failed to survive even in the initial presence of MoCM when EPO was delayed for 48 hours (a mean of 16% of the BFU-E derived colonies in the marrow and a mean of 26% of the BFU-E derived colonies in the blood). Iscove[70] suggested that if EPO is not added when the progenitor matures and develops putative EPO receptors, the EPO receptors are either physically or functionally lost and the progenitor can no longer respond to the hormone. Furthermore, our data suggest that the population of BFU-E responding to EPO at 48 hours is composed of (a) erythroid progenitors that require a factor or factors in MoCM to respond to EPO (ie, the population of BFU-E derived colonies above background when EPO was added initially), and (b) erythroid progenitors that require a factor(s) to survive if EPO addition is delayed (a portion of the population of BFU-E that could respond to EPO initially in the IMDM controls).

Using these conditions and using a selected lot of MoCM as a positive standard control, it was clear that the purified factors were at best only capable of stimulating the growth of a subpopulation of CFU-GM, BFU-E, or CFU-GEM. Sieff and colleagues[7] made similar observations for the activity of GM-CSF. The results of our experiments using target cell populations of marrow fractions separated by velocity sedimentation and marrow populations before and after freezing suggest that the purified factors primarily affected the growth of relatively mature subpopulations of progenitor cells. This observation seems in conflict with the observation that they are able to stimulate subpopulations of blood progenitors, all believed to be of a primitive nature.[43,78] One possible explanation is that subpopulations of circulating progenitor cells exist that may be more easily recruited to respond to stress demands than comparable progenitors in the marrow and, as such, may be more sensitive to the purified factors. This explanation appears compatible with the evidence that GM-CSF, Ppo α, and Ppo can regulate the functional activity of mature neutrophils.[1,4,15,18,76] We are presently further investigating the differences between marrow and blood progenitors.

We found the activities of GM-CSF and Ppo α on the growth of hematopoietic colonies to be indistinguishable, and Ppo α is now believed to be identical to GM-CSF. Both factors were able to promote the growth of neutrophilic, monocytic, and eosinophilic colonies as previously reported.[15,76,79] They were only able to stimulate subpopulations of these morphologically distinct CFU-GM derived colonies when compared with MoCM, however; similar observations have previously been reported for GM-CSF.[7,76] In day 14 cultures stimulated by GM-CSF and Ppo α, most GM colonies were small (<40 cells) and only a rare GM colony reached >100 cells. This was in striking contrast to day 14 cultures stimulated by MoCM which contained only GM colonies >40 cells; >70% of these colonies were several hundred to several thousand cells. Begley and colleagues[80] observed that GM colonies stimulated by semipurified CSF α and CSF β were smaller than GM colonies stimulated with HPCM. Both GM-CSF[1] and Ppo α[15] inhibit neutrophil migration, and we believe that most of the GM colonies (<40 cells) in cultures stimulated by GM-CSF and Ppo α were probably derived from mature CFU-GM and were inhibited from dispersing. To examine this question, we tested the activity of GM-CSF and Ppo α on marrow progenitor cell populations following freezing. The results showed a marked reduction in their ability to stimulate GM colonies from the postfreezing marrow populations, since freezing destroys most of the population of CFU-GM with limited proliferative potential (forming colonies of <40 cells). In addition, in four separate experiments, we examined the activity of GM-CSF on fractions from the Ficoll isokinetic gradient that separates the cells primarily on the basis of cell size. GM-CSF showed the greatest activity on CFU-GM in the most rapidly sedimenting large cell fraction (F13-15) and the least activity on CFU-GM from the slowest sedimenting small cell fraction (F4-10). Most of the activity of GM-CSF on this latter fraction was attributed to contaminating mature CFU-GM that were destroyed on freezing. Most small GM colonies sustained by GM-CSF were derived from mature CFU-GM in the large cell fractions. Only a rare colony reached a size >100 cells as compared with MoCM. Taken together, these results strongly support the suggestion that the subpopulation of GM colonies stimulated by GM-CSF and Ppo α are primarily derived from mature CFU-GM in the marrow.

When EPO was added initially, GM-CSF and Ppo α showed no consistent activity on the growth of BFU-E derived colonies from the marrow, but did have consistent, although variable, activity on the growth of erythroid colonies from the blood. When EPO addition was delayed for 48 hours, however, GM-CSF and Ppo α had similar activity on the growth of BFU-E from marrow and blood (between 20% and 45% of the total BFU-E population stimulated by MoCM). The addition of hemin to the cultures had no obvious effect on these results when the target populations had not been subjected to freezing. GM-CSF showed a reduction in ability to stimulate erythroid colonies from postfreezing marrow populations when hemin was absent, but GM-CSF activity was restored by the addition of hemin. Although hemin improved the size and hemoglobinization of BFU-E derived colonies stimulated by MoCM, the addition of hemin to MoCM cultures did not significantly affect the number of BFU-E. We have no explanation for the effect of hemin on GM-CSF activity on marrow populations following freezing although hemin had a similar effect on the IMDM negative control cultures. Our results clearly demonstrate that GM-CSF and Ppo α do not affect the growth of the majority of the BFU-E populations stimulated by MoCM in either marrow or blood. Further studies will be necessary to define more exactly the nature of their activity on the growth of BFU-E derived colonies and to delineate the subpopulation(s) of BFU-E whose growth they can affect.

GM-CSF and Ppo α also promoted the growth of a subpopulation of CFU-GEM when compared with MoCM. The addition of hemin to the cultures significantly enhanced their ability to stimulate CFU-GEM, but even in the presence of hemin the CFU-GEM stimulated by these two purified factors were almost invariably smaller and fewer than CFU-GEM derived colonies obtained with MoCM. Our experiments showed that GM-CSF had the greatest activity on CFU-GEM in the most rapidly sedimenting large cell fraction (F13-15) and had very little activity on CFU-GEM from the slowest sedimenting small cell fraction (F4-10), which contains most of the CFU-GEM responding to MoCM stimulation (Fig 3 and Table 4). As with CFU-GM and BFU-E, this suggests that GM-CSF is acting primarily on the growth of more mature CFU-GEM derived colonies.

Kerk and co-workers[81] showed that in mouse marrow there are at least two classes of pluripotent hemopoietic progenitor cells that differ in their capacity for self-renewal. The more primitive class of pluripotent progenitor cells was initially quiescent; therefore, initiation of colony development was delayed. This latter class of pluripotent progenitor cells is probably similar to the population of CFU-GEM from human marrow found mainly in the small cell fractions, most of which fail to respond to GM-CSF.

Metcalf and colleagues[76,82] found that activities of GM-CSF were identical to those of semipurified human CSF α, and we found that activities of GM-CSF and Ppo α on the growth of human hematopoietic colonies are indistinguishable. These combined observations strongly suggest that GM-CSF, Ppo α and semipurified CSF α have very similar if not identical properties and may well be the same molecule.

Natural Ppo and rPpo have identical activities when compared on the same marrow population.[19] In the present studies, although we were unable to compare them on the same marrow population, the results using rPpo were in the same range as the results obtained with natural Ppo.

Ppo did have activity on the growth of a subpopulation of CFU-GM from marrow and blood populations. Ppo promoted the growth of neutrophilic and monocytic colonies as previously observed,[14,18,19] but not of eosinophilic colonies. Ppo stimulated a greater number of day 7 GM colonies >40 cells when compared with GM-CSF and Ppo α, but only a portion of the number in cultures stimulated by MoCM. In day 14 cultures stimulated by Ppo, most of the GM colonies were >40 cells, unlike those found with GM-CSF and Ppo α, but they were nevertheless much smaller and fewer in number than in cultures stimulated by MoCM. The ability of Ppo to stimulate GM colonies from postfreezing marrow populations was reduced, and this reduction was primarily due to a loss of a subpopulation of day 7 CFU-GM derived colonies following freezing.

Ppo showed no detectable activity on the growth of colonies derived from BFU-E or CFU-GEM. This observation is in conflict with previously reported studies demonstrating that Ppo stimulates colonies derived from BFU-E and CFU-GEM in nonadherent and nonadherent–T lymphocyte-depleted marrow cells.[14,18,19,83,84] Based on the removal of most of the monocytes and T lymphocytes in these previous studies, the observed stimulatory activity of Ppo on BFU-E and CFU-GEM progenitor cells led to the designation of this growth factor as a pluripoietin growth factor. As we showed, however, when bone marrow or blood cells are rigorously depleted of accessory cells and cloned at very low numbers to minimize further the effects that non–colony-forming cells as well as large numbers of developing GM colonies may have, Ppo shows no direct pluripotent hematopoietic growth activity.

Ppo is now designated as human G-CSF based on: (a) its biological activity on enriched progenitor cell populations as reported in the present studies; (b) the amino acid sequence of cloned Ppo[19] that is almost identical to a cloned human G-CSF reported by Nagata and colleagues[85]; (c) sequence homology with murine G-CSF[86]; and (d) its biological activity as presently described that is similar to semipurified CSF β, which Metcalf[82] has suggested is likely to be a close analogue of murine G-CSF.

EPA showed no detectable activity on either the size or number of colonies derived from CFU-GM, BFU-E, or CFU-GEM. EPA has activity on the growth of both early and late erythroid precursors from buffy coat and light-density human bone marrow cells.[2,12] We and other investigators[7,9] have been unable, however, to detect significant BPA activity of EPA on enriched populations of human progenitor cells in high serum culture conditions. EPA may act through accessory cells.

Pledger and colleagues[87-89] proposed a competence-progression model in which growth factors can be divided into those that induce a state of competence (competence factor), in which state the target cells can then respond to a second growth factor (progression factor). They showed that multiple growth factors are necessary to stimulate nonhematopoietic cell types maximally. Goustin and colleagues[90] recently reviewed several of these growth factors for nonhematopoietic cells as well as a group of colony-stimulating factors (CSF-1, CSF-2, multi-CSF) for hematopoietic cells and suggested that a similar model of multiple growth factors may be necessary to stimulate hematopoietic cell types maximally. Metcalf[82] recently reviewed the molecular biology and functions of GM-CSF and showed that multiple factors are necessary to stimulate the proliferation of all available GM progenitor cells.[76,82] Quesenberry[91] reviewed a new class of hematopoietic growth factors that have no intrinsic ability to stimulate hematopoietic colonies alone but that act synergistically with other growth factors to enhance their effects and suggested that synergizing activities may act as either competence or progression factors in concert with other regulators.

Our results show that GM-CSF (Ppo α) can promote the growth of subpopulations of CFU-GM, BFU-E, and CFU-GEM, and that G-CSF (Ppo) can promote the growth of a subset of CFU-GM. Our data suggest that all three factors mainly affect the growth of relatively mature subpopulations of progenitor cells. Our results clearly show that additional factor(s) exist in MoCM that are necessary to stimulate the total populations of these progenitor cells in vitro. How these factors may fit into a competence-progression model cannot be determined until other factors can be shown to act synergistically with these factors to enhance their activities. We have begun to examine selected factors for synergistic activities and will report our results when the experiments have been completed.

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CONCISE REPORT

Glycoprotein Ib and Glycoprotein IX Are Fully Complexed in the Intact Platelet Membrane

By Xiaoping Du, Lucinda Beutler, Changgeng Ruan, Peter A. Castaldi, and Michael C. Berndt

Two new murine monoclonal antibodies, AK 1 and SZ 1, reactive with the human platelet glycoprotein (GP) Ib–IX complex have been produced by the hybridoma technique. Both AK 1 and SZ 1 immunoprecipitated the GP Ib–IX complex from Triton X-100-solubilized, periodate-labeled platelets. With trypsinized, labeled platelets, AK 1, SZ 1, and FMC 25 (epitope on GP IX) immunoprecipitated a membrane-bound proteolytic fragment of the GP Ib–IX complex consisting of GP IX and an ≈25,000 mol wt remnant of the α-chain of GP Ib disulfide-linked to the β-subunit. Unexpectedly, although AK 1 and SZ 1 immunoprecipitated purified GP Ib–IX complex, neither antibody immunoprecipitated the individual components of this complex, GP Ib or GP IX. When GP Ib and GP IX were recombined, however, AK 1 and SZ 1 again immunoprecipitated the reformed complex, strongly suggesting that both antibodies were recognizing an epitope present only on the intact complex. Cross-blocking studies indicated that AK 1 and SZ 1 recognized a very similar or identical epitope that was proximal to the epitope for FMC 25. Both AK 1 and SZ 1 bound to a similar number of binding sites (≈25,000) on intact platelets as monoclonal antibodies directed against either GP Ib or GP IX. The combined data suggests that GP Ib and GP IX are fully complexed in the intact platelet membrane.

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THE ADHESION of platelets to exposed vascular subendothelium constitutes the initial event in hemostasis. At high shear flow, platelet adhesion becomes von Willebrand factor-dependent and requires the binding of von Willebrand factor to a specific adhesion receptor on platelets, the glycoprotein (GP) Ib–IX complex.[1] The GP Ib–IX complex consists of two components, GP Ib (mol wt 170,000) and GP IX (mol wt 22,000).[2] Besides its requirement for the von Willebrand factor-dependent adhesion of platelets, recent evidence suggests that GP Ib plays an important role in other platelet-associated receptor functions such as the response of platelets to α-thrombin[3,4] and as one of the target antigens for autoantibody in idiopathic thrombocytopenic purpura.[5,6] GP IX is important for the recognition of platelets by quinine/quinidine drug-dependent antibodies.[7] Previous evidence from our laboratory indicates that both GP Ib and GP IX are tightly complexed in detergent solution since both GPs copurify,[2] coimmunoprecipitate with either anti-GP Ib or anti-GP IX monoclonal antibodies,[2,7] and occur in the same immunoprecipitin arc on crossed immunoelectrophoresis.[2] The degree of association that exists between these two GPs in the intact platelet membrane was not clear, however. In this article, we describe two anti-GP Ib–IX complex monoclonal antibodies, AK 1 and SZ 1, that are directed against complex-specific epitopes. Both these antibodies bind to a similar number of binding sites on intact platelets as monoclonal antibodies directed against either GP Ib or GP IX. The combined data suggest that GP Ib and GP IX are fully complexed in the intact platelet membrane.

MATERIALS AND METHODS

Monoclonal antibodies. The monoclonal antibodies were prepared according to our previously described methods[2,8] using 8-week-old BALB/C mice and either washed human platelets (SZ 1, IgG_{2a}) or purified GP Ib–IX complex (AK 1, IgG_1; AK 3, IgG_{2a}) as the immunogens. Hybridoma selection, cloning, production of ascites, and purification of murine monoclonal IgG were all performed as previously described.[2,8] Purified AK 1 and SZ 1 IgG stored in 0.01 mol/L of Tris, 0.15 mol/L of sodium chloride, pH 7.4, were radioiodinated by the chloramine T method; >95% of the incorporated radioactivity was precipitable by 10% (wt/vol) trichloroacetic acid. Other monoclonal antibodies against the human platelet GP Ib–IX complex have been previously reported: FMC 25 (IgG_1)[7,9]; AN 51 (IgG_1)[7,10]; SZ 2 (IgG_1)[8]; and WM 23 (IgG_1).[2,7] In immunoprecipitation experiments, SZ 21 (IgG_1) directed against the human platelet GP IIb/IIIa complex and FMC 18 directed against an antigen on *Toxoplasma gondii* were used where appropriate as negative controls.[8] Purified FMC 25 and WM 23 IgG were coupled to Affigel 10 (Bio-Rad, Richmond, CA) according to the manufacturer's instructions.

Platelet preparation and labeling. Platelet preparation from freshly drawn blood, platelet washing, and the periodate-labeling of platelets were each performed as previously described.[11] Labeled platelets (10^9/mL) were treated at 22°C for 15 minutes with 10 μg/mL of trypsin (Boehringer Mannheim, Sydney, Australia). At 15 minutes the reaction was terminated by making the digest 0.2 mmol/L in phenylmethanesulfonyl fluoride, 200 μg/mL in leupeptin, and 200 μg/mL in soybean trypsin inhibitor (all inhibitors from Sigma, St Louis). The platelets were then washed once by centrifugation.

Purification of tritium-labeled GP Ib–IX complex, GP Ib, and GP IX. The platelets from 10 U of platelet concentrate obtained within 24 hours of venepuncture (Parramatta Red Cross Blood Transfusion Service) were washed as previously described,[2] suspended at ~4 × 10^9 platelets/mL in 100 mL of 0.01 mol/L of Hepes buffer (0.15 mol/L in sodium chloride, 5 mmol/L in EDTA), pH

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7.4, and equilibrated at 0°C in a melting ice bath. The platelet suspension was made 1 mmol/L in sodium periodate (Univar grade, Ajax Chemicals, Sydney, Australia), left 10 minutes in the dark at 0°C, washed once by centrifugation at 1,000 g for 15 minutes, and resuspended to its original volume in the same buffer. The platelets were treated for 10 minutes with 400 mCi of sodium (^{3}H)borohydride (70 Ci/mmol; Amersham, Sydney, Australia) and washed once by centrifugation. Platelet membrane extraction and purification of GP Ib–IX complex on WM 23-Affigel 10 were then performed as previously described.[2] Labeled GP Ib was separated from labeled GP IX by chromatography on FMC 25-Affigel 10.[2] To ensure that the purified GP Ib and GP IX were totally devoid of the other GP, the purified GP Ib and GP IX were mixed overnight with either 1 mL of FMC 25-Affigel 10 (anti-GP IX) or 1 mL of WM 23-Affigel 10 (anti-GP Ib), respectively. GP Ib and GP IX were allowed to recomplex by mixing together equivalent counts of each purified labeled GP (excess GP IX)[9] at 4°C for 12 hours (both GPs in 0.01 mol/L of Tris, 0.15 mol/L of sodium chloride, 0.001 mol/L of EDTA, 0.1% (vol/vol) Triton X-100, 0.02% (wt/vol) sodium azide, pH 7.4).

Monoclonal antibody binding to platelets. Binding was assessed with radioiodinated AK 1 IgG or SZ 1 IgG at 22°C using platelet-rich plasma prepared from venous blood collected into 3.2% sodium citrate as anticoagulant as previously described in detail.[2,12] Binding was measured at 60 minutes as it did not alter after this time. Cross-blocking experiments were performed as previously described[8] and examined the effect of a 50-fold excess of the anti-GP Ib–IX complex monoclonal antibodies, AN 51, SZ 2, WM 23, AK 3, FMC 25, AK 1, and SZ 1, on the equilibrium binding of 1 μg/mL of either radioiodinated AK 1 or SZ 1 to platelets. Similar experiments were performed to examine the effect of 150 μg/mL of affinity-purified rabbit anti-GP Ib$_\alpha$ or nonimmune rabbit IgG on the equilibrium binding of 1 μg/mL of either radioiodinated AK 1 or SZ 1 to platelets. Because the rabbit antibody agglutinated platelets, these experiments were performed with washed, formaldehyde-fixed platelets.[8] The rabbit anti-GP Ib$_\alpha$ antibody was raised as previously described[2] and affinity purified on GP Ib-Affigel 10.[2] Bound antibody was eluted with 0.1 mol/L of glycine, pH 2.4; the eluted fractions were immediately neutralized by the addition of one-fifth vol of 1 mol/L of Tris, pH 8.5, and then dialyzed exhaustively against 0.01 mol/L of Tris, 0.15 mol/L of sodium chloride, pH 7.4.

General methods. Immunoprecipitation with murine anti-GP Ib–IX complex monoclonal antibodies, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein staining, fluorography, and Western blot analysis were all performed as previously described.[2,7]

RESULTS

Immune studies: Demonstration that the anti-GP Ib–IX complex monoclonal antibodies, AK 1 and SZ 1, are directed against complex-specific epitopes. AK 1 and SZ 1 are murine monoclonal IgG antibodies that were raised using either purified GP Ib–IX complex or washed human platelets as the respective immunogens. AK 1 and SZ 1 both immunoprecipitated the components of the GP Ib–IX complex from the Triton X-100 lysate of periodate-labeled platelets (data not shown) as well as purified, tritium-labeled GP Ib–IX complex (Fig 1, lanes 5 and 9; GP Ib, nonreduced mol wt 170,000; GP IX, nonreduced mol wt 22,000). The purified, tritium-labeled GP Ib–IX complex (lane 1) and the AK 1 and SZ 1 immunoprecipitates derived from this complex (lanes 5 and 9) also contained two minor bands of slightly lower mol wt than GP Ib. As previously discussed in detail,[7] these bands probably derived from partial reduction of GP Ib either as a consequence of the borohydride reduction labeling procedure or experimental manipulation. The band of 22,000 mol wt was confirmed to be GP IX since electrophoresis under reducing conditions clearly distinguished this band from the β-subunit of GP Ib (mol wt 25,000) (data not shown). With trypsin-treated platelets, AK 1, SZ 1, and FMC 25 (epitope on GP IX)[2,7] immunoprecipitated a membrane-bound proteolytic fragment of the GP Ib–IX complex consisting of GP IX and a nonglycosylated remnant of the α-chain of Gp Ib (mol wt 25,000) that was disulfide-linked to the β-subunit of GP Ib (data not shown).[7] In an attempt to define the epitope specificity of AK 1 and SZ 1 further, we evaluated their reactivity by performing Western blot analysis after SDS-PAGE of purified GP Ib–IX complex under either nonreducing or reducing conditions. Although this technique readily demonstrated that FMC 25 was directed against GP IX, both AK 1 and SZ 1 were unreactive. As an alternative approach, we performed immunoprecipitation using purified tritium-labeled GP Ib–IX complex, GP Ib, and GP IX (Fig 1). Surprisingly, although both AK 1 and SZ 1 immunoprecipitated the GP Ib–IX complex, neither antibody precipitated either GP Ib (lanes 6 and 10) or GP IX (lanes 7 and 11). When GP Ib was allowed to recomplex with GP IX, however, both AK 1 and SZ 1 again immunoprecipitated the reconstituted complex (lanes 8 and 12), strongly suggesting that both AK 1 and SZ 1 only recognized an epitope(s) on the intact GP Ib–IX complex. In this experiment, the AK 1 and SZ 1 immunoprecipitates of the reconstituted complex (lanes 8 and 12) had a lower ratio of counts for GP Ib relative to GP IX[9] than did the immunoprecipitates derived from the original complex (lanes 5 and 9). This finding was variable, and in duplicate experiments the ratios were similar whether the immunoprecipitates were derived from either the original or reconstituted complex (not shown). Although the cause of this phenomenon is unclear, it may represent variable aggregation of GP IX as a consequence of the low pH treatment required for its isolation.[2] In control experiments (not shown), SZ 21 directed against the platelet membrane GP IIb/IIIa complex (as a negative control) did not immunoprecipitate the GP Ib–IX complex, GP Ib, GP IX, or the reformed complex; AK 3, directed against an epitope on the α-chain of GP Ib as determined by Western blot analysis, immunoprecipitated the GP Ib–IX complex, GP Ib, and the reformed complex; and FMC 25, directed against an epitope on GP IX, immunoprecipitated the GP Ib–IX complex, GP IX, and the reformed complex. The latter two results indicate that the failure of either AK 1 or SZ 1 to immunoprecipitate GP Ib or GP IX was not due to a general loss of antigenicity of either glycoprotein as a result of complex dissociation and support the probability that AK 1 and SZ 1 are directed against complex-specific epitopes.

Binding of AK 1 and SZ 1 to platelets. Binding of radioiodinated AK 1 and SZ 1 to platelets in platelet-rich plasma was rapid, being 50% complete within ≈5 minutes and complete within 30 to 60 minutes. In a typical experiment (Fig 2), the amount of bound antibody increased linearly with the concentration of added antibody and

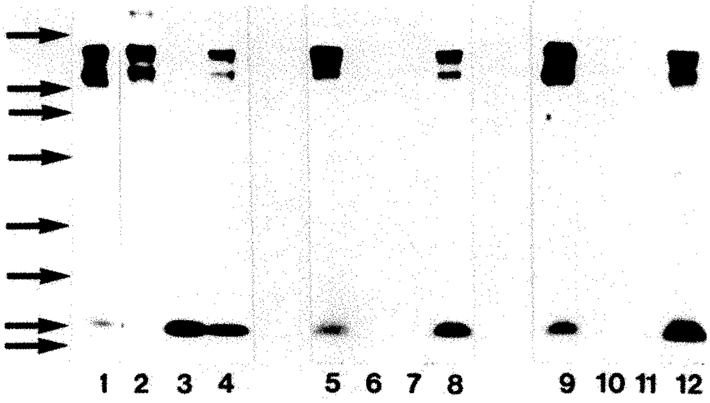

Fig 1. Fluorography of an exponential 5% to 15% sodium dodecyl sulfate-polyacrylamide gel run under nonreducing conditions of the AK 1 (lanes 5 through 8) and SZ 1 immunoprecipitates (lanes 9 through 12) derived from either tritium-labeled glycoprotein (GP) Ib-IX complex (lane 1), tritium-labeled GP Ib (lane 2), tritium-labeled GP IX (lane 3), or tritium-labeled reformed complex (lane 4), respectively. Lane 1 is one-quarter of the exposure of lanes 2 through 12. In order of decreasing mol wt, mol wt markers are myosin (200,000), β-galactosidase (130,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000).

reached saturation at ~0.6 μg of bound antibody/10^8 platelets. As previously found with other anti-GP Ib–IX complex monoclonal antibodies,[2] this type of binding curve is not amenable to Scatchard analysis but does allow calculation of the number of immunoglobulin binding sites per platelet from the plateau region of the platelet–antibody titration curve (assuming that antibody binding is monovalent). The results of platelet binding studies with radioiodinated AK 1 and SZ 1 are summarized in Table 1 along with previous binding data for other anti-GP Ib–IX complex monoclonal antibodies. Both AK 1 and SZ 1 bound to an essentially identical number of binding sites on platelets as were observed with monoclonal antibodies directed against either GP Ib or GP IX, suggesting that essentially all GP Ib and GP IX in the intact platelet membrane was in complex form.

Cross-blocking studies indicated that AK 1 and SZ 1 were directed against an identical or very similar epitope(s) since each antibody completely blocked the binding of the other. FMC 25 directed against an epitope on GP IX blocked the binding of AK 1 and SZ 1 by ≈25% and ≈30%, respectively. Conversely, AK 1 and SZ 1 blocked the binding of FMC 25 by ≈50% and ≈80%, respectively, indicating that the epitope for FMC 25 was sterically adjacent to the epitope(s) for AK 1 and SZ 1 and that, in agreement with the immunoprecipitation results, all three antibodies mapped into the membrane-associated region of the GP Ib–IX complex.[7] In contrast, AN 51, WM 23, AK 3, and SZ 2 directed against the glycocalicin region of the α-chain of GP Ib[2,7,8] (this study), and affinity-purified rabbit anti-GP Ib_α IgG, did not block the binding of either AK 1 or SZ 1 to platelets. Affinity-purified rabbit anti-GP Ib_α IgG inhibited the binding of FMC 25 to platelets by ≈35%.

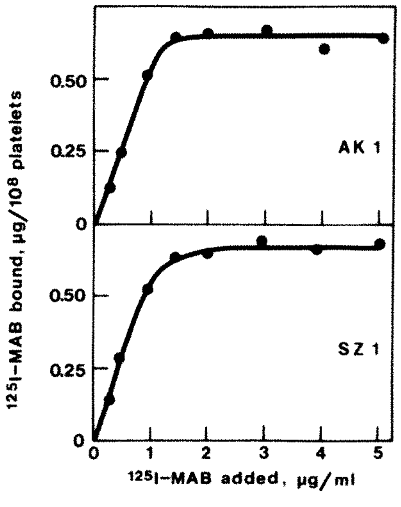

Fig 2. Effect of increasing concentrations of ^{125}I-labeled AK 1 and SZ 1 on their specific, steady-state binding to platelets in platelet-rich plasma. The binding analysis was performed as previously described.[2] MAB, monoclonal antibody.

DISCUSSION

In 1983, in a reexamination of the GP abnormalities in Bernard-Soulier syndrome platelets, we found that two platelet membrane glycoproteins, GP Ib and GP IX, were both apparently genetically absent in this disorder and suggested that this phenomenon might be due to the normal tight association of these two GPs in the intact human platelet membrane.[9] Although there is strong evidence that GP Ib and GP IX are tightly associated in detergent solution[2,7] and that an approximately equal number of copies of both GPs exists on intact platelets,[2] the degree of association that exists between these two GPs in the platelet membrane has remained unclear. This has been an important area requiring resolution since both GPs are involved in various key aspects of platelet function.[1,3-7]

In this study, we have described two new anti-GP Ib–IX complex monoclonal antibodies, AK 1 and SZ 1, that appear to be directed against complex-specific epitopes. Although both antibodies immunoprecipitated the GP Ib–IX complex, neither antibody immunoprecipitated either GP Ib or GP IX.

Table 1. Binding of Anti-Glycoprotein Ib-IX Complex Monoclonal Antibodies to Platelets

Antibody	Epitope	Number of Binding Sites (x ± SD)
AK 1	Ib/IX	22,800 ± 1,200 (n = 4)
SZ 1	Ib/IX	24,000 ± 2,200 (n = 4)
AN 51*	Ib	22,000 ± 2,700 (n = 3)
WM 23*	Ib	21,000 ± 3,400 (n = 3)
FMC 25*	IX	20,100 ± 2,700 (n = 3)

Binding analysis was performed as described in the Materials and Methods section.

*Data from ref. 2, with permission.

When the separated GPs were recombined, however, both AK 1 and SZ 1 again immunoprecipitated the reconstituted complex (Fig 1), strongly suggesting that both antibodies reacted only with an epitope(s) expressed on the intact complex.* Further, cross-blocking studies suggest that AK 1 and SZ 1 are directed against an identical or closely related epitope that is sterically adjacent to the epitope for FMC 25 on GP IX. Monoclonal and polyclonal antibodies against the α-chain of GP Ib did not affect the binding of AK 1 and SZ 1 to platelets. With trypsin-treated platelets, AK 1, SZ 1, and FMC 25 all immunoprecipitated GP IX and an ~25,000 mol wt nonglycosylated remnant of the α-chain of GP Ib that remained disulfide-linked to the β-subunit. AK 1 and SZ 1 therefore map into the membrane-associated region of the GP Ib–IX complex, which would be expected potentially to contain complex-specific antigenic sites. In this regard, the binding of quinine/quinidine drug-dependent antibodies to platelets is also apparently complex-specific and is also blocked by FMC 25.[7]

AK 1 and SZ 1 bound to an essentially identical number of binding sites on platelets, as did monoclonal antibodies directed against either GP Ib or GP IX (Table 1), strongly suggesting that all GP Ib and GP IX in the platelet membrane is in complex form. Although binding of AK 1 and SZ 1 to platelets could possibly displace normally dissociated GP Ib and GP IX toward complex, this is unlikely since both AK 1 and SZ 1 bound to platelets with a similar time course as other anti-GP Ib or anti-GP IX monoclonal antibodies. Furthermore, affinity-purified rabbit anti-GP Ib_{α} IgG inhibited the binding of the anti-GP IX monoclonal antibody, FMC 25, to platelets by ≃35%, a result in accord with significant interaction between GP Ib and GP IX in the platelet membrane.

Considerable evidence now shows that GP Ib plays an important functional role in the von Willebrand factor-dependent adhesion of platelets to exposed vascular subendothelium[1] and in the α-thrombin activation of platelets[3,4] and that GP IX is important for quinine/quinidine drug-dependent antibody platelet recognition.[7] The combined data from this and previous studies suggest that these functions are mediated at the platelet surfaced by a heterodimer complex of GP Ib and GP IX.

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*The term "complex-specific" is not meant to imply that the epitope is necessarily physically located at the junction of the complex. The epitope(s) for AK 1 and SZ 1 could be present on either GP IX or the membrane-associated fragment of GP Ib, the conformation of the epitope(s) only being preserved in the intact complex.

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CONCISE REPORT

Glucose-6-Phosphate Dehydrogenase of Malaria Parasite *Plasmodium falciparum*

By Akira Yoshida and Eugene F. Roth, Jr

Plasmodium falciparum growth is impaired in glucose-6-phosphate dehydrogenase (G6PD)-deficient red blood cells (RBCs), and malaria has been implicated in the spreading of deficient variants in malaria-endemic areas. Recent reports suggest that the malaria parasite can adapt itself to grow in these variant RBCs by producing its own G6PD, but studies on parasite G6PD are very limited. In this report, we define the properties of the parasite G6PD. G6PD was partially purified from infected and uninfected variant RBCs associated with severe G6PD deficiency. G6PD from infected RBCs contained two components separable by starch gel electrophoresis: a major component (~90% activity) with a very slow anodal electrophoretic mobility and a minor component (~10% activity) with the same mobility as the host G6PD. Parasite G6PD exhibited much higher affinity (low K_m) to G6P and nicotinamide-adenine dinucleotide phosphate (NADP) than did human G6PD. Southern blot hybridization indicated that the parasite genome contained nucleotide sequences that were hybridizable with the human G6PD cDNA. These data indicate that the parasite is capable of adapting to G6PD-deficient RBCs by producing its own G6PD.

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GENETIC DEFICIENCY of the X chromosome-linked glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: nicotinamide-adenine dinucleotide phosphate [NADP] oxidoreductase, EC 1.1.1.49; G6PD) is common among the populations of present and past malaria-endemic areas. For example, Gd A(−), associated with 10-15% of normal red cell G6PD activity, is found in 10% to 20% of black populations; and Gd Mediterranean B(−), associated with a more severe deficiency, is commonly found among Mediterranean, Southeast Asian, and South Pacific populations. It is generally accepted that malaria has been a major selective pressure for the spread of these common deficiency variants in these populations. In vivo and in vitro growth of *Plasmodium falciparum* has been found to be retarded in G6PD-deficient red blood cells.[1,2] In red blood cells, formation of reduced glutathione (GSH) depends solely on nicotinamide-adenine dinucleotide phosphatase (NADPH) generated by the hexose monophosphate shunt pathway. A lower GSH level in G6PD-deficient red cells drastically impairs adenosine triphosphate (ATP)-phosphoribosyl-transferase (EC 2.4.2.17) activity, presumably causing insufficient production of 5′-phosphoribosyl-1-pyrophosphate to permit parasite growth.[3]

Early studies on animal malaria parasites (*P lophurae, P berghei*, and *P knowlesi*) indicated that the parasites demonstrated Embden-Meyerhof pathway activity, but no measurable activity of the hexose monophosphate shunt pathway.[4-6] Recent reports, however, have suggested that *P falciparum* contains its own G6PD, and can adapt itself to grow in G6PD-deficient red blood cells.[7-9] This paper reports the nature of G6PD produced by *P falciparum* and the existence of nucleotide sequences in the parasite genome that are hybridizable with the human G6PD cDNA.

MATERIALS AND METHODS

P falciparum (FCR-3 strain), grown in normal human red blood cells (obtained from Drs S. Schulman and J. Vanderberg, New York University School of Medicine), was cultured in an in vitro system described by Jensen and Trager.[10] G6PD-deficient blood was obtained from a Persian-Jewish male donor described in the previous studies.[9] The parasites were continuously grown in the G6PD-deficient red cells, and were enriched for trophozoite/schizont stages by gelatin sedimentation, which generally provided parasitemias of from 40% to 85%.[11] The parasite-enriched cells were washed with isotonic phosphate-buffered saline, containing 10 mmol/L glucose, and kept frozen at −70°C.

For partial purification of G6PD, the red blood cells (infected and uninfected host cells and control normal cells) were hemolyzed with 2 vol of 5 mmol/L phosphate, pH 6.4, containing 0.1 mmol/L NADP, and 0.1 volume of toluene. The stroma-free hemolysate was treated with a diethyl aminoethyl (DEAE)-cellulose (DE-22 Whatman, Kent, England) column, as previously described, to eliminate hemoglobin and other contaminants.[12] The enzyme was precipitated with ammonium sulfate (40 g/100 mL at pH 6.4); redissolved in an adequate amount of 0.01 mol/L phosphate, pH 7.0, containing 10 µmol/L NADP and 1 mmol/L 2-mercaptoethanol; and dialyzed against the same buffer. The G6PD activity was spectrophotometrically assayed.[12]

Starch gel electrophoresis was performed, as previously described, and the enzyme was visualized by G6PD-activity staining.[13]

For the study of the parasite genome, DNA was prepared from the infected host red blood cells (about 10 mL) following the previous method[14] with some modifications. Briefly, DNA was extracted from the cells with 9 vol of 0.1 mol/L Tris-HCl, pH 7.4, containing 12.5 mmol/L EDTA, 1% sodium dodecylsulfate, and proteinase K (500 µg/mL), at 37°C for two hours. DNA was purified by stepwise treatments with chloroform-phenol, chloroform-isoamylalcohol, phenol, and ether, and precipitated with ethanol. Approximately 800 µg of parasite DNA was obtained. The human DNA was prepared from leukocytes by the previous method.[14]

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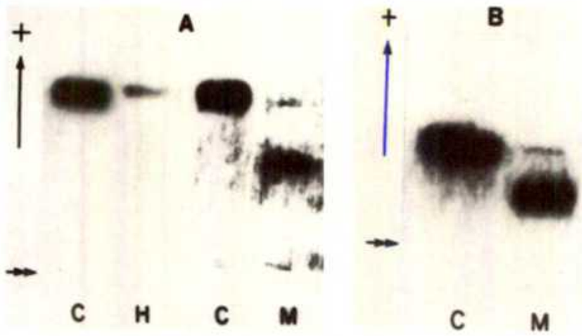

Fig 1. Electrophoresis of human G6PD and malaria-parasite G6PD. Starch gel electrophoresis was performed using the Tris-EDTA-borate buffer system, pH 8.6 (A), and using the phosphate buffer system, pH 7.0 (B). Samples: C, control G6PD B(+); H, G6PD from uninfected host RBCs; M, G6PD from parasite-infected host RBCs.

The genomic DNA samples were digested by restriction endonucleases (EcoR I, Hind III, Pst I, or Msp I), and the digests were separated by electrophoresis in 0.8% agarose gel and transferred onto a nitrocellulose filter.[15] The filter was hybridized with the ^{32}P-labeled cDNA probes, as previously described.[16]

RESULTS AND DISCUSSION

The anodal electrophoretic mobility of the parasite G6PD was much slower than either the host human G6PD or the control normal G6PD B(+) at pH 8.6 and at pH 7.0 (Fig 1). Judging from the relative intensity of activity staining, more than 90% of the activity can be attributed to the parasite G6PD and the minor remainder to the host G6PD in the partially purified enzyme prepared from the parasite-infected host red cells.

The G6PD activity of the parasite-infected host red cells was 0.23 units/g hemoglobin, whereas that of the uninfected host red cells was 0.01 units and that of the control normal red cells was 6.2 units. The kinetic properties (K_m values for G6P and NADP, and the rate of utilization of substrate analogues) are shown in Table 1. Parasite G6PD exhibits very low K_m for both G6P and NADP; therefore, parasite G6PD is more active than human G6PD in red blood cells, where G6P and, particularly, NADP concentrations are lower than the K_m values of the human G6PD. The pH optima of parasite G6PD was found to be 7.5 to 9.0, which is similar to that of the normal G6PD B(+).

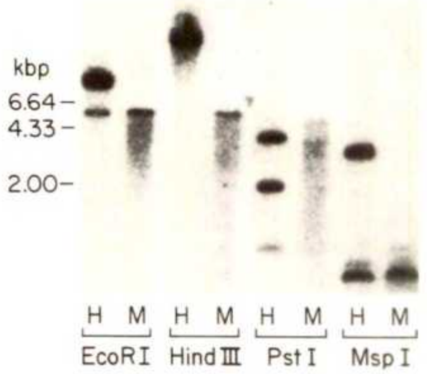

Fig 2. Southern blot hybridization of human DNA and malaria-parasite DNA. Samples: H, human DNA; M, parasite DNA.

The electrophoretic mobility and kinetic properties of the host G6PD are the same as those reported for G6PD Mediterranean B(−), ie, the male donor is a Gd B(−) hemizygote.

Southern blot hybridization analysis indicated that the parasite genome contained nucleotide sequences that were homologous to the human G6PD cDNA (Fig 2). The parasite's DNA was found to be hybridizable also with the human alcohol dehydrogenase cDNA, aldehyde dehydrogenase cDNA, and aldolase A cDNA, but not with the human phosphoglycerate kinase (PGK) (X-linked) cDNA (Southern blot hybridization patterns are not shown). Since PGK is one of the most evolutionally conservative proteins[17] and a key enzyme for ATP generation in the glycolytic pathway, the lack of nucleotide sequences hybridizable with the PGK cDNA in the parasite's genome was rather unexpected.

Hemplemann and Wilson reported that the putative *P falciparum* G6PD migrated as a slow-moving band,[7] while, according to Usanga and Luzzatto, the parasite G6PD had the same electrophoretic mobility as normal human G6PD B(+).[8] The enzymatic properties of the putative parasite G6PD were not reported. The present study clearly indicates that the parasite G6PD has very slow anodal electrophoretic mobility and exhibits unique kinetic properties. The discrep-

Table 1. Properties of *P falciparum* G6PD

Enzyme	Electrophoretic Mobility		K_m (μmol/L)		Analogue Utilization (%) 2-d G6P/deamino NADP/	
	pH 8.6	pH 7.0	G6P	NADP	G6P	NADP
Normal B (+)	100	100	55	4	3.5	60
Mediterranean B (−)	100	100	19-26	1.2-1.6	23-27	300-400
Host G6PD	100	100	27	1.4	30	380
Malaria G6PD	63	55	11	0.8	10	110

Enzyme activity was assayed in a reaction mixture containing 0.1 mol/L Tris-HCl, pH 8.0, 10 mmol/L $MgCl_2$, 0.6 mmol/L G6P (or 2-deoxy G6P), and 0.2 mmol/L NADP (or deamino NADP) at 25 °C. The values of analogue utilization are the ratio (%) of enzyme activities assayed in the presence of natural substrates (ie, G6P and NADP) and in the presence of the substrate analogues (ie, 2-deoxy G6P and deamino NADP). K_m values were determined using the reaction mixtures containing various concentrations of G6P of NADP.[12] Electrophoretic mobility is expressed taking that of normal G6PD B(+) as 100. Values of Mediterranean G6PD B(−) are based on previous data.[18] Contamination of host G6PD in the malaria G6PD preparation is less than 10% (Fig 1).

ancy could be due to the difference in the malaria parasite strains used.

In conclusion, *P falciparum* can produce its own G6PD in G6PD-deficient host red blood cells. The parasite-G6PD activity of the infected deficient cells (measured in vitro under optimal assay conditions) reaches about 5% of the activity of normal red cells. Parasite G6PD has a high affinity to the substrates, and could be physiologically more active than human G6PD. The parasite may sustain its own growth and is capable of adapting to G6PD-deficient red blood cells by producing its own G6PD.

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CONCISE REPORT

von Willebrand Factor Released From Weibel-Palade Bodies Binds More Avidly to Extracellular Matrix Than That Secreted Constitutively

By Lee Ann Sporn, Victor J. Marder, and Denisa D. Wagner

Large multimers of von Willebrand factor (vWf) are released from the Weibel-Palade bodies of cultured endothelial cells following treatment with a secretagogue (Sporn et al, *Cell* 46:185, 1986). These multimers were shown by immunofluorescent staining to bind more extensively to the extracellular matrix of human foreskin fibroblasts than constitutively secreted vWf, which is composed predominantly of dimeric molecules. Increased binding of A23187-released vWf was not due to another component present in the releasate, since releasate from which vWf was adsorbed, when added together with constitutively secreted vWf, did not promote binding. When iodinated plasma vWf was overlaid onto the fibroblasts, the large forms bound preferentially to the matrix. These results indicated that the enhanced binding of the vWf released from the Weibel-Palade bodies was likely due to its large multimeric size. It appears that multivalency is an important component of vWf interaction with the extracellular matrix, just as has been shown for vWf interaction with platelets. The pool of vWf contained within the Weibel-Palade bodies, therefore, is not only especially suited for platelet binding, but also for interaction with the extracellular matrix.

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VON WILLEBRAND FACTOR (vWf) mediates platelet adhesion to the vessel wall by forming a bridge between glycoprotein Ib on the platelet surface and an as yet unidentified component of the vascular subendothelium.[1] Endothelial cells synthesize vWf[2] as a series of disulfide-bonded multimers that range in size from 0.5 to 20 million daltons.[1] Predominantly small multimers of vWf are secreted constitutively, and the large multimeric forms are stored in the Weibel-Palade bodies.[3] The contents of the Weibel-Palade bodies can be released from cultured endothelial cells upon exposure to nonphysiologic secretagogues such as the calcium ionophore A23187 and phorbol myristate acetate,[4,5] or physiologic stimuli such as thrombin[4,6] and fibrin.[7] For several reasons, the large multimers of vWf are considered to be functionally important: First, only the largest vWf multimers are specifically missing from the plasma of patients with type IIa von Willebrand disease,[8] and second, the largest multimers of vWf have a higher affinity for glycoprotein Ib than do the low molecular weight forms.[9-11] In addition, we now show that the large multimers of vWf found within the Weibel-Palade bodies bind the most avidly to the extracellular matrix, the in vitro counterpart of the basement membrane.

MATERIALS AND METHODS

Cell culture and labeling. Endothelial cells were obtained from human umbilical veins as previously described[12,13] and grown in McCoy's 5a medium (Flow Laboratories, McLean, VA) containing 20% undialyzed fetal bovine serum. Human foreskin fibroblasts (HFF) in fourth passage were a gift from Sue Schmidt (Infectious Disease Unit, University of Rochester), and were grown under the same conditions. For continuous metabolic labeling, cell cultures were grown in the same medium in the presence of [^{35}S]-methionine (25 μCi/mL, 1132 Ci/mmol) (New England Nuclear, Boston, MA). Cells were treated with the calcium ionophore A23187 (Sigma, St Louis) using a solution of 10 μmol/L A23187 in McCoy's 5a medium with 20% fetal bovine serum. Iodinated plasma vWf was a generous gift from Dr Philip Fay (Hematology Unit, University of Rochester) and was prepared as described previously.[14]

Antisera. The preparation and characterization of monospecific antisera against human vWf used for immunofluorescence were previously described.[11,12] For vWf purification, antiserum was purchased from Calbiochem-Behring (La Jolla, CA). Immunofluorescence staining using anti-vWf antiserum was carried out as previously described.[12]

Purification of vWf and gel electrophoresis. Cells were lysed and vWf was immunopurified as described previously.[15] Two-percent agarose gels were prepared with 0.1% sodium dodecyl sulfate (SDS) in 0.05 mol/L phosphate, pH 7.0; running buffer was 0.1 mol/L phosphate buffer, pH 7.0, with 0.1% SDS.

Adsorption of vWf from A23187 releasate. Protein A-sepharose (Sigma) (100 mg/mL of sample) was preincubated with anti-vWf antiserum (100 μL/mL of sample) then washed three times with McCoy's 5a medium. Releasate was shaken with this mixture for 1.5 hours at room temperature, Sepharose beads were centrifuged and the supernatant used for the HFF overlay experiment described in the text.

RESULTS AND DISCUSSION

This study compares binding of vWf secreted constitutively and that released from Weibel-Palade bodies to the extracellular matrix of HFF. Prior to conducting binding studies, we have tested the stability of the various multimeric forms of vWf while in contact with HFF for an extended incubation period. Endothelial cells were metabolically labeled with [^{35}S]-methionine for three days and culture

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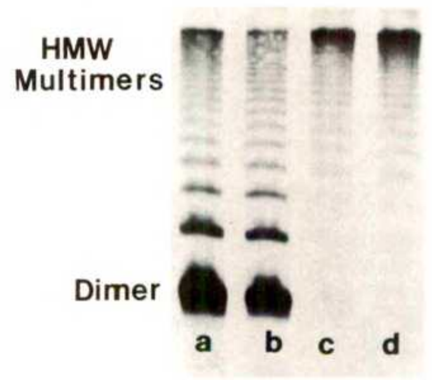

Fig 1. Multimeric composition of vWf used in matrix-binding experiments. vWf secreted constitutively (a, b) or released by A23187 (c, d) from metabolically-labeled endothelial cells both after (b, d) or before (a, c) a 24-hour incubation with HFF was immunopurified and electrophoresed on a 2% agarose gel. Constitutively secreted vWf contains a broad range of multimers, but predominantly dimer molecules, while that released from cells by A23187 treatment is highly enriched in the high molecular weight (HMW) vWf multimers. There was no significant change in multimeric composition of vWf after incubation over HFF for 24 hours.

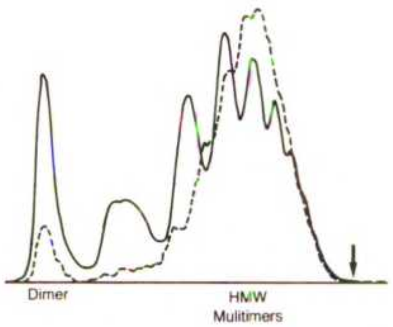

Fig 3. The multimeric pattern of vWf bound to HFF. Iodinated plasma vWf (starting material) was overlaid onto a confluent flask of HFF for 48 hours, at which time the HFF were washed extensively and lysed. Samples of both starting material and cell lysate were electrophoresed, nonreduced on the same 2% agarose gel and submitted to autoradiography. The densitometric scan of starting material (——) is from a short exposure of the gel, and the scan of bound vWf is from a longer exposure (---). The figure is provided as a comparison of multimeric profiles and is not representative of relative quantities of vWf in the two samples. Arrow points to the origin of the gel.

medium containing constitutively secreted vWf was removed. Following a six-hour chase period in unlabeled culture medium, cells were subjected to A23187 treatment for ten minutes. Half of each sample was then placed on a confluent flask of HFF for 24 hours and the remaining half incubated under the same conditions but in the absence of cells. vWf was immunopurified from all samples. Figure 1 compares the multimeric pattern of vWf secreted constitutively (A, B) and released by A23187 (C, D) with and without incubation with HFF. This experiment indicated that the presence of the HFF did not alter the multimeric pattern or cause significant degradation of vWf.

The comparative study of binding of constitutively secreted vWf and A23187-released vWf to the extracellular matrix of HFF was then conducted as follows. Human umbilical vein endothelial cells metabolically labeled for three days were subjected to A23187 treatment for ten minutes. The released material was aged for 24 hours in a tissue culture flask at 37°C so that the multimers were of comparable age with those secreted constitutively by cultures labeled continuously for 24 hours. vWf was immunopurified from both samples. The 24-hour medium sample and the A23187 releasate contained approximately the same number of counts per minute in vWf per volume; therefore, the two samples contained a similar number of vWf subunits but in different multimeric forms (Fig 1). Equal volumes of these media from unlabeled identical flasks prepared in parallel were then overlaid onto cultures of HFF grown on glass coverslips. After 72 hours incubation on the HFF, the coverslips were washed extensively, and stained by immunofluorescence using anti-vWf antiserum. Figure 2A represents control cells cultured in the absence of vWf, treated in the same manner and showing no matrix staining. The medium sample containing the constitutively secreted vWf produced only a trace of matrix decoration (Fig 2B). In contrast, the vWf released by A23187 produced a strong fluorescent signal (Fig 2C). The strength of the fluorescent signal produced by purified plasma vWf has been shown to be dependent on the vWf concentration (weight/volume) in the culture medium.[16] Since equal concentrations of vWf were placed on HFF, it appeared that the A23187-released vWf bound more avidly to the extracellular matrix.

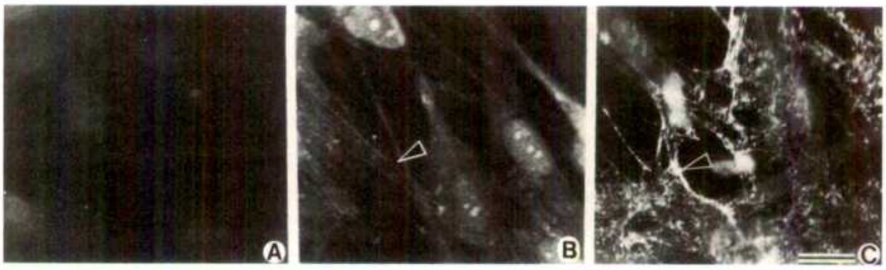

Fig 2. Comparison of binding to extracellular matrix of vWf secreted constitutively and released by A23187. vWf secreted without stimulation over a 24-hour period (B) and an equivalent amount of A23187-released vWf (C) were placed on HFF grown on glass coverslips. (A) represents control fibroblast cultures to which no vWf was added. After 72 hours, the coverslips were fixed, permeabilized, and stained by fluorescence with anti-vWf antiserum. Arrowheads point to extracellular matrix filaments decorated with vWf. A23187-released vWf produced a much stronger fluorescence signal indicating that it bound more extensively. Bar = 20 μm.

To address the possibility that a component other than vWf in the A23187 releasate enhanced binding of the protein to the extracellular matrix, a mixing experiment was performed (not shown). Culture medium containing constitutively secreted vWf was mixed with A23187 releasate, from which vWf was removed by adsorption (described in Materials and Methods) and then incubated with the HFF. The presence of the vWf-depleted A23187 releasate did not cause an increase in fluorescent signal in comparison to controls that were mixed with fresh medium only. This experiment does not, however, rule out the possibility that a yet unknown modification exists in the vWf stored within the Weibel-Palade bodies that could cause enhanced binding to the matrix. Most likely the difference in affinity for the extracellular matrix of constitutively secreted vWf and A23187-released vWf is due to differences in multimeric size (ref 3, Fig 1). This idea is supported by the study of small multimeric forms of vWf synthesized by endothelial cells in the presence of monensin or ammonium chloride. These small multimers do not incorporate into the extracellular matrix of endothelial cells, nor do they bind to the matrix of HFF as seen by immunofluorescent staining with anti-vWf antiserum.[17,18]

For more direct evidence that the large multimeric sizes of vWf bind better to the extracellular matrix, vWf purified from plasma and containing all multimeric forms was iodinated and placed on HFF culture. After 48 hours, media was removed and cells were washed extensively and lysed. To check the efficiency of our lysing procedure, an equivalent amount of cold vWf was placed on HFF grown on glass coverslips; the cells were lysed, and the coverslip was stained by fluorescence with anti-vWf antiserum. As no staining was observed, this revealed that the lysing procedure removed all cellular material and extracellular matrix. Without further processing of the samples, a small amount of both iodinated starting material used for the HFF overlay and cell lysate were electrophoresed, nonreduced on a 2% agarose gel. A comparison of the densitometric scans of vWf in starting material and of bound vWf revealed that the bound vWf was highly enriched in the high molecular weight (HMW) multimers (Fig 3). When the starting material and bound vWf were compared reduced by analysis on polyacrylamide gels, no apparent change in the subunit size was observed indicating that it was not significantly degraded or covalently crosslinked by a transglutaminase to another extracellular matrix component (not shown).

Since the multimers of vWf are composed of many identical subunits arranged in a linear array,[19] enhanced binding of the large multimers could be due to multiple interactions along the length of the vWf molecule with available binding sites in the extracellular matrix. The notion that the polyvalency of the multimers of vWf is important to its function is not new. Low-molecular weight forms of vWf exhibit enhanced ristocetin cofactor activity following their adsorption to colloidal gold.[20] This indicates that dimers and other small multimers possess the potential for interaction with platelets, but this potential is manifested only following formation of larger multimers. The importance of polyvalency has been reported for other adhesive systems as well. For example, the species-specific reaggregation of cells in the marine sponge *Microciona prolifera* is mediated by a proteoglycanlike aggregation factor. High-affinity interaction between cells could be reconstituted by chemical crosslinking of fragments of the aggregation factor containing low-affinity cell binding sites.[21]

In our previous studies, we have shown that the vWf released from the Weibel-Palade bodies is highly enriched in the largest multimeric forms of vWf synthesized by the cells.[3] The present observations reveal that this same pool of molecules is not only best suited for interaction with platelets, but also for interaction with the extracellular matrix, the in vitro model of the basement membrane.

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CONCISE REPORT

Platelets Express a Membrane Protein Complex Immunologically Related to the Fibroblast Fibronectin Receptor and Distinct From GPIIb/IIIa

By Filippo G. Giancotti, Lucia R. Languino, Adriana Zanetti, Giuseppe Peri, Guido Tarone, and Elisabetta Dejana

We have previously identified and characterized a membrane glycoprotein complex (GP150/135) that functions as fibronectin receptor (FN-R) in fibroblast adhesion. Here we report that an immunologically related protein complex is expressed at the surface of human platelets. Antibodies monospecific for the smaller subunit (GP135) of the fibroblast FN-R in fact specifically stained the platelet surface, as determined by FACS analysis, and reacted with a component of molecular weight (mol wt) 138,000 as shown in western blots of platelet membranes. Moreover, the same antibodies precipitated the 138,000 component together with a 160,000 protein, suggesting that the two molecules are associated in a supramolecular complex. A comparative analysis indicated that this protein complex is distinct from the GPIIb/IIIa complex, known to function as a receptor of wide specificity for fibrinogen, fibronectin, and von Willebrand factor. Differential extraction experiments revealed that the platelet 138,000 component is an integral membrane protein.

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PLATELETS PLAY a crucial role in hemostasis by adhering to the exposed subendothelial matrix and interacting with each other to form the large aggregates that constitute the hemostatic plug. These events are regulated by a family of platelet adhesive proteins: fibrinogen, fibronectin, and von Willebrand factor. While fibronectin and von Willebrand factor are able to support the adhesion and spreading of platelets to the subendothelial matrix, fibrinogen induces aggregation by interacting with the membrane glycoprotein complex GPIIb-IIIa.[1] Recently it has been shown that this complex can also bind fibronectin[2,3] and von Willebrand factor,[4] suggesting that platelets have a common binding site for the interaction with a variety of proteins. This interaction occurs via the Arg-Gly-Asp sequence that was initially identified as the cell attachment site of fibronectin, but is also present on fibrinogen and von Willebrand factor.[5] Arg-Gly-Asp–containing peptides indeed compete for the binding of fibronectin, fibrinogen, and von Willebrand factor to platelets[6,7] and also inhibit adhesion of platelets to fibronectin.[8,9]

Fibronectin interaction with GPIIb/IIIa does not fully elucidate the mechanism of platelet adhesion and spreading with regard to this protein. Unactivated platelets, which cannot bind fibronectin through GPIIb/IIIa,[10] still maintain normal adhesion,[8] suggesting the existence of a second binding site for fibronectin on the platelet surface.

We have previously described a membrane glycoprotein of 135,000 mol wt (GP135) that, by the criteria of function and codistribution, appears to be the mouse fibroblast fibronectin receptor (FN-R).[11,12] This molecule participates in a complex with another glycoprotein of approximately 150,000 mol wt and interacts with the cell-binding domain of fibronectin via an Arg-Gly-Asp sequence (Giancotti et al, manuscript in preparation). Here we report that an immunologically related protein complex exists on the surface of human platelets and that it is distinct from the well-characterized GPIIb/IIIa complex.

MATERIALS AND METHODS

Preparation of the anti–FN-R serum and monospecific GP135 antibodies. To obtain an antiserum to the fibronectin receptor complex, rabbits were immunized with the material immunoprecipitated with the anti–baby hamster kidney (anti-BHK) serum[11] from the surface of 5 × 10^8 SR-BALB cells. This material consisted mainly of the two glycoproteins GP135 and GP150, which were shown to be involved in cell adhesion to fibronectin.[11,12] Monospecific antibodies to GP135 were obtained from this antiserum by affinity chromatography on GP135-Sepharose. Briefly, GP135 was purified from mouse tumors by lentil lectin–Sepharose chromatography followed by separation on an hydroxylhapatite column. The purification, monitored by immunoblotting with the anti–FN-R serum, yielded about 200 μg of protein out of 20 g of starting material. The molecule was 90% pure and GP135 was the only antigenic component recognized by the anti–FN-R serum in this fraction (the detailed procedure will be published elsewhere).

Preparation of platelets and platelet plasma membranes. For FACS analysis, human platelets were isolated from freshly drawn blood as previously described.[13] Plasma membranes were prepared according to Jennings et al[14] from outdated platelet concentrates. Rat platelets were obtained from 40 mL of fresh blood collected into acid-citrate-dextrose by differential centrifugation, followed by gel filtration on Sepharose CL 2B. Extraction of peripheral and integral membrane proteins was performed as described.[15]

Fluorescence flow cytometric analysis. Antibody binding to the surface of human and rat platelets was determined by fluorescence flow cytometric analysis. Gel-filtered platelets, collected from fresh blood, were stained using the following procedure: 100 μL of platelet suspension containing 250,000 platelets/μL were incubated with the antibodies for 30 minutes at 37°C. Platelets were then washed in phosphate-buffered saline (PBS) and incubated for 30 minutes at 37°C with fluorescein-isothiocyante (FITC)-conjugated swine $F(ab')_2$ antirabbit IgG. After extensive washings with PBS, platelets (1 × 10^4 for each assay) were analyzed by flow cytometry using a

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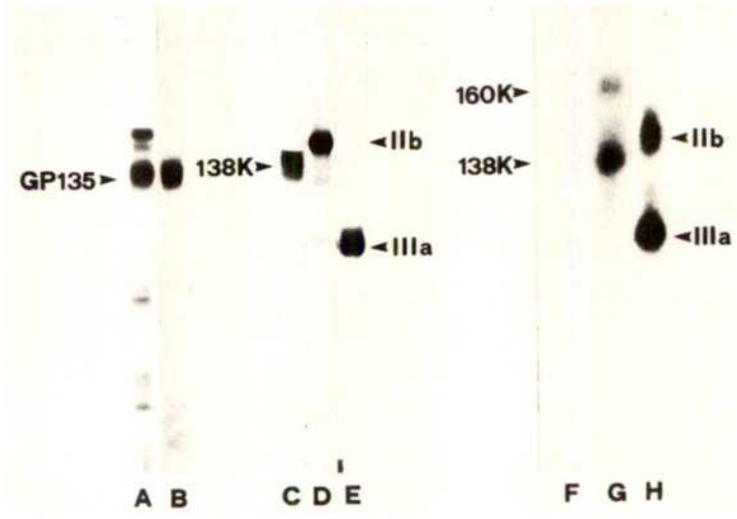

Fig 1. Identification of the platelet 160/138 complex. Lane A: membrane proteins immunoprecipitated from the cell surface of ^{35}S-methionine–labeled SR-BALB mouse fibroblasts with anti–FN-R serum. Lanes B through E: membrane proteins from SR-BALB mouse fibroblasts (B) or human platelets (C through E) separated by SDS-PAGE, transferred to nitrocellulose paper, and probed with affinity-purified GP135 antibodies (B, C), PMI-1 (D) and B2.12 (E) monoclonal antibodies. Lanes F through H: proteins immunoprecipitated from 125-I–labeled platelet plasma membrane fraction with normal rabbit IgG (F), affinity-purified GP135 antibodies (G) and anti-IIb/IIIa serum (H). All the samples were run on a 5% to 10% polyacrylamide gradient gel under nonreducing conditions. Apparent mol wts were deduced from standards.

FACS 440 (B-D FACS systems, Becton Dickinson, Sunnyvale, CA) with an 80-μm orifice. The gate was empirically set to detect cells of 0.5- to 3-μm size and exclude larger cells and subcellular debris. Leukocytes representing less than 2% of the total cell population were therefore excluded from the analysis. No platelet aggregates were present in the suspension after antibody staining.

Immunoblotting and immunoprecipitation of platelet membrane proteins. Immunoblotting analyses were performed on plasma membrane fractions separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes, as previously described.[12] Monospecific GP135 antibodies were used at 40 μg/mL. Monoclonal antibodies to GPIIb (PMI-1)[16] and GPIIIa (B2.12)[17] were used at 10 μg/mL. The anti-IIb/IIIa serum[18] was used 1:50. PMI-1 and rabbit anti-IIb/IIIa were kindly donated by E.F. Plow and M.H. Ginsberg, Research Institute of Scripps Clinic, La Jolla, CA; B2.12 was obtained from P. Thiagarajan, University of Washington, Seattle. Bound antibodies were revealed by peroxidase-labeled secondary antibodies according to the manufacturer's instructions. Preparation of the fibroblast crude membrane fraction for immunoblotting was also described previously.[12] For immunoprecipitation analysis, an aliquot of platelet plasma membranes was solubilized with 0.5% Triton X-100 (Pierce, Rockford, IL) in PBS plus $CaCl_2$ 1 mmol/L and $MgCl_2$ 1 mmol/L and proteins were iodinated with 125-I by the chloramine T method. The iodinated sample was then subjected to immunoprecipitation. Immunoprecipitation of fibroblast FN-R complex was performed as described.[11,12]

RESULTS

The anti–FN-R serum immunoprecipitates from SR-BALB mouse fibroblasts two major glycoproteins of 150,000 (GP150) and 135,000 (GP135) apparent mol wt in SDS-PAGE under nonreducing conditions (Fig 1, lane A). To determine whether a molecule immunologically crossreactive with the FN-R was present at the platelet surface, monospecific antibodies to the lower mol wt component, GP135, were prepared by affinity chromatography (see Materials and Methods) and used throughout this study. Their specificity was demonstrated by western blotting on fibroblast membrane proteins (Fig 1, lane B).

Results of the fluorescence flow cytometry indicated that platelets were positive for GP135 antibody surface staining (Table 1). About 17% of human and 86% of rat platelets reacted with GP135 antibodies. Anti-IIb/IIIa serum, used as a positive control, stained about 60% of the platelets in both cases. A unimodal distribution of fluorescence intensity was observed with human and rat platelets with both antibodies, indicating a single cell population. The relatively low percentage of GP135-positive human platelets can probably be explained by the fact that antibodies raised to mouse GP135 tend to show a weaker reaction with the human than with the rodent glycoprotein. The reactivity of GP135 antibodies with platelets was also confirmed by immunofluorescence microscopy.

To identify the molecule recognized by GP135 antibodies on human platelets, western blotting and immunoprecipitation analysis were performed. As shown in Fig 1 (lane C), the antibodies reacted with a platelet plasma membrane component of approximately 138,000 mol wt. When antibodies were used to immunoprecipitate Triton-solubilized, 125-I–labeled platelet plasma membranes, a 160,000–mol wt component was detected in addition to the 138,000 protein (Fig 1, lane G). Since the 160,000 molecule does not contain antigenic determinants recognized by the GP135 antibodies, as shown by the immunoblotting assay (Fig 1, lane C) and by

Table 1. Expression of the GP135-Related Molecule at Platelet Surface

	Percent Cells Positive			
Cells	α-GP135	NIIg	α-GpIIb/IIIa	NIS
Human platelets	17 ± 1	1 ± 1	60.4 ± 4	8.7 ± 2
Rat platelets	86 ± 4	2 ± 1	54.4 ± 5	5.8 ± 2
Red blood cells	4 ± 1	3 ± 1	5 ± 2	6 ± 3

Platelets were prepared and analyzed by fluorescence flow cytometry as described in Materials and Methods. Cell staining was performed using GP135 antibodies (α-GP135) or nonimmune rabbit immunoglobulins (NIIg) at a final concentration of 40 μg/mL. Anti-GpIIb/IIIa rabbit serum (α-GpIIb/IIIa) and nonimmune rabbit serum (NIS) were used at 1/25 dilution. Red blood cells were used as negative control. Values are expressed as mean percentage of three determinations ± SD.

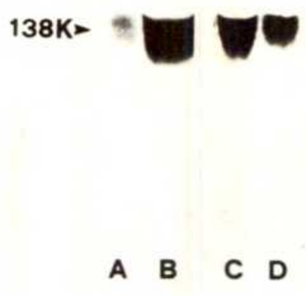

Fig 2. Membrane association of the platelet 138,000 protein. Platelet plasma membranes were extracted with 0.1 mol/L NaOH or 0.5% Triton X-100. Proteins recovered in the soluble and insoluble fractions were separated by SDS-PAGE, transferred to a nitrocellulose paper, and reacted with affinity-purified GP135 antibodies. Lane A: NaOH-soluble fraction. Lane B: NaOH-insoluble fraction. Lane C: Triton X-100–soluble fraction. Lane D: Triton X-100–insoluble fraction.

analogy with the fibroblast FN-R, we concluded that this molecule may be coprecipitated due to its association with the 138,000 component.

The 160/138 protein complex was found to be distinct from the GPIIb/IIIa heterodimer. In fact, western blotting (Fig 1, lanes D and E) and immunoprecipitation (Fig 1, lane G) experiments indicated that both GPIIb and GPIIIa have electrophoretic mobilities distinct from those of the 160,000 and 138,000 molecules. Moreover, with the antisera used, no immunological cross reaction between these components was observed.

Association of the 138,000 molecule with the plasma membrane was studied by extracting membrane pellets with either 0.1 mol/L NaOH (pH 12) or 0.5% Triton X-100, under conditions allowing the selective solubilization of peripheral or integral proteins, respectively.[15] As shown in Fig 2 (lanes A, B), alkaline treatment did not extract significant amounts of the 138,000 molecule, indicating that it is not a peripheral membrane protein. On the other hand, the nonionic Triton X-100 detergent was able to solubilize the protein to a considerable extent (Fig 2, lane C), suggesting its integral nature. A small but significant amount of 138,000 protein remained associated with the detergent-insoluble fraction (Fig 2, lane D) consisting mainly of cytoskeletal proteins.[20] These data support the idea that the platelet 138,000 molecule is somehow associated with the submembranous cytoskeleton.

DISCUSSION

The results here presented demonstrate that platelets express a cell surface protein complex related to the FN-R of fibroblastic cells. The platelet complex consists of two polypeptide chains of mol wts 160,000 and 138,000, associated in a noncovalent manner. These two subunits are likely to be structurally distinct, as indicated by the fact that monospecific GP135 antibodies react with the 138,000 polypeptide, but not with the 160,000 polypeptide.

Previous experiments with GP135 antibodies indicate that both fibroblasts and hemopoietic cells of the erythroid and myeloid lineages express the FN-R molecule.[20] The molecules expressed on fibroblast (GP135), hemopoietic cells (GP145), and platelets (138,000) are immunologically cross-reactive and therefore similar. The differences in mol wts might reflect differences in the extent of glycosylation or in the primary sequences.

Another cell surface complex involved in adhesive interactions, the platelet GPIIb/IIIa, has been found on a number of different cell types.[18] Recent data indicate that the GPIIb/IIIa and FN-R complexes belong to a family of structurally related proteins that interact with their ligands via the Arg-Gly-Asp recognition sequence.[5] The data presented in this paper indicate that both these receptors are expressed at the platelet surface.

Fibronectin is likely to be important in platelet adhesion to the subendothelium as suggested by Houdijk et al.[21] One fibronectin binding site on platelets is represented by the glycoprotein complex GPIIb/IIIa.[2,3] Binding of fibronectin to this receptor can be completed by physiological concentrations of fibrinogen,[22] suggesting that fibronectin–GPIIb/IIIa interaction is unlikely to occur in plasma. Moreover, GPIIb/IIIa glycoproteins become competent for fibronectin binding only after activation of platelets by thrombin,[10] a condition that is not necessary for their adhesion and spreading on fibronectin.[8] Taken together, this evidence suggests that a surface binding site distinct from GPIIb/IIIa may be involved in platelet–fibronectin interaction. Existence of more than one receptor for a single ligand is not an uncommon event. Fibroblasts seem to have two distinct binding sites for fibronectin[23] and platelets are able to bind von Willebrand factor by the GPIIb/IIIa complex,[4] as well as by the GPIb glycoprotein.[24] In this paper we demonstrate that platelets, in addition to the GPIIb/IIIa heterodimer, express a distinct cell surface 160/138 complex. This molecule, structurally related to the fibroblast FN-R, represents a potential second binding site for fibronectin on platelets.

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CONCISE REPORT

Detection of Hemophilia A Carriers Using Intragenic Factor VIII:C DNA Polymorphisms

By R.L. Janco, J.A. Phillips, III, P.J. Orlando, M.J. Woodard, K.L. Wion, and R.M. Lawn

A DNA polymorphism for an *XbaI* site in intron 22 of the human factor VIII:C gene extends the utility of DNA methods for carrier detection in families segregating for hemophilia A. While the DNA polymorphism detected by a *BclI* site in intron 18 of the factor VIII:C gene was informative for 41% of females studied, the *BglI*/intron 25 polymorphism provided no additional information because of apparent linkage disequilibrium. In contrast, the *XbaI* intron 22 polymorphism was useful in 53% of women who were uninformative (homozygous) for either the *BclI* or *BglI* polymorphisms. Using the *BclI*/intron 18 and *XbaI*/intron 22 intragenic polymorphisms, we could provide highly accurate information for 68% of women we studied who were at risk for carriership. The carrier status of the remaining 32% could be determined utilizing the closely linked *TaqI*/St14 DNA polymorphism.

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HEMOPHILIA A, an X-linked disorder producing absence or dysfunction of the factor VIII:C molecule, is the most common severe congenital coagulopathy.[1] Despite advances in home infusion therapy, hemophilia A remains a potentially crippling and life-threatening condition. Therapy-induced complications include chronic hepatitis, inhibitor formation, and infection with the human immunodeficiency virus (HIV).[2] Accordingly, many women at risk for being hemophilia carriers seek genetic counseling. Carrier detection (phenotype assignment) by statistical analysis of factor levels and pedigree probabilities is limited in its applicability to certain cases and includes significant risk for error, since misclassification rates vary from 5% to 15%.[3-6] The discovery of restriction fragment length polymorphisms (RFLPs) within and closely linked to the factor VIII:C gene have recently provided markers for linkage analysis to determine the inheritance of normal or mutant (hemophilia) factor VIII:C genes. Use of these RFLPs enables carrier detection and prenatal diagnosis to be accomplished more accurately by actual genotype rather than phenotype assessment. Once the linkage phase for intragenic RFLPs is established, carrier assignment is basically nonprobabilistic if the consultand is heterozygous for the RFLP. Unfortunately, a single intragenic DNA polymorphism with two (+ and −) alleles can be informative for no more than 50% of women as predicted by the Hardy-Weinberg law.[7] Additional intragenic RFLPs, if informative, may serve to extend this highly accurate approach to more women at risk for transmitting the hemophilia gene.

We have compared the utility of three intragenic RFLPs (*BclI*/intron 18, *XbaI*/intron 22, and *BglI*/intron 25) with a highly polymorphic, closely linked *TaqI*/St14 RFLP.[8,9] Our results suggest a strategy for providing accurate genotype assignment for virtually all female relatives of hemophiliacs in an economical, efficient way.

MATERIALS AND METHODS

Study group. Informed consent was obtained from normal volunteers and obligate carriers in accordance with policies of the Vanderbilt Committee for the Protection of Human Subjects. Leukocyte DNA samples from women at risk for transmitting the hemophilia A gene were obtained and the status of the closely-linked *TaqI*/St14 and intragenic *BclI*/intron 18 RFLPs were determined for 106 and 93 women, respectively. Forty-three women from 29 families had additional analyses performed for the *BglI*/intron 25 and *XbaI*/intron 22 RFLPs. Of these 43, 16 were relatives of nine sporadic cases. Samples needed to establish the linkage phase were obtained from affected males and other family members.

Isolation of DNA and Southern transfer. Whole venous blood (12 to 15 mL) was drawn using EDTA as an anticoagulant. Leukocyte DNA was isolated as previously described.[9] DNA was digested with various restriction endonucleases according to the manufacturer's instructions and subjected to electrophoresis for 16 hours at 25 volts in 1% agarose slab gels. DNA fragments were transferred to filters (Gene Screen Plus membranes, New England Nuclear, Boston MA) by Southern blotting.[10]

DNA probes. Probes were radiolabeled with [^{32}P] dATP and [^{32}P] dCTP. Hybridization and autoradiography were carried out as previously described.[9] The DNA sequences used in these studies were genomic fragments in exon 17-18, intron 22, exon 26, and St14 (anonymous, closely linked).[8] Exon 26 analyses were kindly done by Dr Stylianos Antonarakis (Johns Hopkins University School of Medicine, Baltimore, MD) and the St14 clone was a gift from Prof J.L. Mandel, Strasbourg, France. The DNA probes for exon 17-18 and intron 22 are available through the NIH Human Repository, ATCC, Rockville, MD.

RESULTS AND DISCUSSION

Frequency of informative RFLPs. A woman was considered to be informative if she was heterozygous for an RFLP and the linkage phase of the DNA polymorphism and the normal or abnormal factor VIII:C allele could be established. The extragenic *TaqI*/St14 RFLP, which is closely linked, was informative in 103/106 (97%) of women studied.

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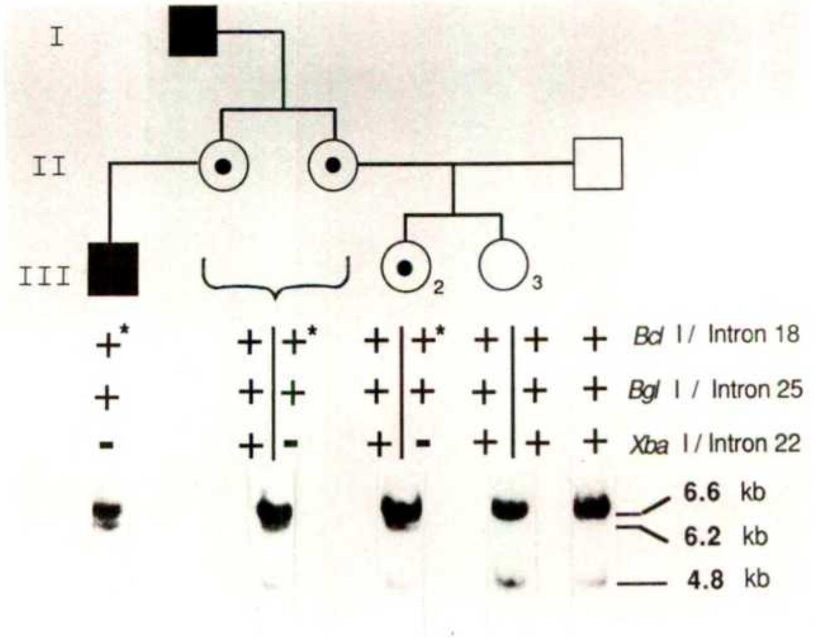

Fig 1. Utility of *Xba*I (intron 22) RFLP in a family uninformative with either the *Bcl*I (intron 18) or the *Bgl*I (intron 25) RFLPs: ■, affected male; ⊙, carrier; ○, normal female; *, haplotype associated with the abnormal (hemophilia) factor VIII:C allele. The symbols + and − indicate the presence or absence of a polymorphic restriction site, respectively. The autoradiograms of DNAs digested with *Xba*I and *Kpn*I and hybridized to the intron 22 probe are shown below. Note that individual III_2 has inherited the − allele and is thus a carrier, while III_3 has inherited two + alleles and is thus normal.

In contrast, the *Bcl*I/intron 18 RFLP was informative for only 38/93 (41%) of women. This latter result is in close agreement with the prediction of Gitschier et al[11] of 42% heterozygosity from estimated allelic frequencies of 71% and 29% ($2\,pq = 2 \times 0.71 \times 0.29 = 0.41$).

The *Bgl*I/intron 25 RFLP was informative for 14/44 women studied (32%). This agrees with prior estimates of heterozygosity that ranged from 11% to 38%.[12]

The *Xba*I polymorphic site in intron 22 was informative for 25/43 women (58%). The proportion of observed heterozygosity was slightly higher than the 48% expected from calculations based on previous reported allelic frequencies of 59% and 41% ($2\,pq = 2 \times 0.59 \times 0.41 = 0.48$).[13]

Linkage disequilibrium between the intron 18 and intron 25 RFLPs. In a prior report, we noted that the intron 25 RFLP offered little additional information to that provided by the intron 18 RFLP.[9] Among 44 females for whom we have analyzed both RFLPs, 14 were informative with both, three were informative with only the intron 18 RFLP, and 27 were not informative with either. A 2 × 2 contingency table testing for association of the two RFLPs suggests the intron 18 and intron 25 RFLPs are in strong linkage disequilibrium ($X^2 = 32.6$, $p < 0.0001$). Thus in our experience the intron 25 RFLP seems to have limited utility when compared to the intron 18 RFLP.

Added utility of the intron 22 RFLP. In contrast to the *Bgl*I/intron 25 RFLP, the *Xba*I/intron 22 RFLP was often informative in those women who were homozygous for the *Bcl*I/intron 18 RFLP (Fig 1). Among 38 women uninformative for the *Bcl*I/intron 18 RFLP, 20 (53%) were informative with *Xba*I/intron 22. Similarly, among 35 women uninformative with *Bgl*I/intron 25, 19 (54%) were informative with the *Xba*I/intron 22. These data suggest that the *Xba*I/intron 22 RFLP may be informative in about 1/2 of those females who are homozygous for the other two intragenic RFLPs. Assuming complete linkage equilibrium, analysis of the *Bcl*I/intron 18 and *Xba*I/intron 22 RFLPs should be informative for about 72% of the females ($41\% + .53 \times 59\% = 72\%$); however, results obtained in earlier studies demonstrated that these polymorphisms are not in complete equilibrium.[13] In our study, this combination was informative for 25/39 (64%) women for whom we had complete data, suggesting that analysis of these two RFLPs could enable highly accurate carrier detection for about ⅔ of female relatives of males with hemophilia A. Moreover, our results suggest the optimal strategy for applying DNA techniques to both carrier detection and prenatal diagnosis of hemophilia A is to utilize analysis of the intron 18 and intron 22 RFLPs.

Uninformative with intragenic RFLPs. Of the 14 of 39 women who were uninformative with any of the three intragenic RFLPs studied, all were informative with the *Taq*I/St14 RFLP. Although there is a significant (3.85%) risk of recombination between this RFLP and the factor VIII:C gene (I. Peake, personal communication, January 1987), results obtained can be used to establish carriership with diagnostic accuracy that likely exceeds that obtained through conventional factor analysis.[3-6]

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CONCISE REPORT

Expression of c-*fos*, c-*myb*, and c-*myc* in Human Monocytes: Correlation With Monocytic Differentiation

By Jay Lee, Kapil Mehta, Mark B. Blick, Jordan U. Gutterman, and Gabriel Lopez-Berestein

Terminal differentiation of human monocytic leukemia cells (THP-1 cells) was associated with the induction of *c-fos*, the down regulation of c-*myb*, and no significant change in the level of c-*myc* expression. Gamma interferon, which resulted in a slight decrease in c-*myb* but no change in c-*fos* or c-*myc* expression, had a transient antiproliferative effect without a morphological or functional differentiation of THP-1 cells. Resting human peripheral blood monocytes have a high c-*fos*, a low c-*myc*, and no detectable c-*myb* expression. These findings suggest that a switch in c-*fos*/c-*myb* expression is associated with the terminal differentiation of cells of the monocytic lineage.

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PROTO-ONCOGENES seem to be involved in the regulation of cellular growth and differentiation.[1-7] Recent studies suggest that c-*fos* may play a role in human and murine monocytic differentiation.[8-10] In fibroblasts, c-*fos* expression is normally very low but is rapidly induced by peptide growth factors,[11,12] suggesting that c-*fos* also plays a role in fibroblast proliferation. The human monomyelocytic leukemia cell line HL-60 can be induced by the tumor promoter 12-0-tetradecanoyl-phorbol-13 acetate (TPA) to differentiate into macrophagelike cells.[13-15] This effect is accompanied by a reduction in the level of c-*myb* mRNA expression[16] and an increase in the level of c-*fos* expression and synthesis of c-*fos* protein.[9,17] The function of the c-*fos* gene product may be associated with the transduction of nuclear signals that, depending on the differentiated state of the cell, may affect regulatory pathways either in the control of proliferation or in the induction or progression of differentiation.

We and others have shown that c-*myb* and c-*myc* are transcribed preferentially in immature hematopoietic cells and that both genes appear to be regulated during myeloid cell differentiation and proliferation.[18-21] Decreased expression of c-*myc* has been demonstrated during the differentiation of the murine myeloid leukemia cell line WEHI-3B[10] and in HL-60,[22] as well as in the terminal differentiation of cultured mouse erythroleukemia (MEL) cells.[23] Down regulation of c-*myb* expression has also been reported during T cell differentiation,[24-25] during the promonocytic stage of myeloid progenitor cells,[26] and during TPA-induced monocytic differentiation of a human myeloblastic leukemia cell line, ML-1.[27] In this study we demonstrate that the terminal differentiation of the human monocytic cell line (THP-1) is associated with a switch of c-*fos* and c-*myb* expression.

MATERIALS AND METHODS

Human peripheral blood monocytes (HPBM) were isolated by centrifugal elutriation as described earlier.[28] The THP-1 cell line was originally derived from a patient with acute monocytic leukemia and is maintained in RPMI 1640 medium, supplemented with 10% porcine serum, 10 mmol/L HEPES buffer, 100 μg/mL streptomycin, and 100 U/mL penicillin.[29] RNA was prepared by the guanidinium cesium chloride method.[30] Polyadenylated RNA was selected by chromatography on oligo(dT)-cellulose columns.[31] The polyadenylated RNA (5 μg/lane) was size-fractionated by electrophoresis in 1.1% agarose gels containing 2.2 mol/L formaldehyde and transferred onto nitrocellulose filters with 20 × standard saline citrate (SSC; 0.15 mol/L sodium chloride and 0.015 mol/L sodium citrate). The filter paper was hybridized to ^{32}P-radiolabeled DNA probes at 42°C for 12 hours by a modification of the method described earlier.[32] The probes were radiolabeled by nick translation to a specific activity of 1 to 3 × 10^8 cpm/μg of DNA. The filters were then washed in 0.1 × SSC and 0.1% sodium lauryl sulfate at 60°C for 60 minutes, air-dried, and autoradiographed with intensifying screens for two to seven days at −70°C. Molecular cloning and preparation of the v-*fos*, c-*myc*, c-*myb*, and β-actin probes were carried out as described previously.[33-36]

RESULTS

The human monocytic leukemia cell line THP-1 differentiates to functionally nonproliferating adherent macrophage-like cells upon exposure to all-*trans*-retinoic acid (RA) or TPA but not to human recombinant interferon-γ (R-IFN-γ).[37] We studied the expression of c-*fos*, c-*myb*, and c-*myc* in normal HPBM, in untreated THP-1 cells, and in THP-1 cells treated with RA (1 μmol/L), TPA (50 ng/mL), or R-IFN-γ (200 U/mL) for 24, 48, and 72 hours. By northern blot analysis, we detected a high level of c-*fos*, a low level c-*myc*, and no c-*myb* expression in freshly isolated HPBM (Fig 1). In contrast, we detected low levels of c-*fos* but a high level of c-*myb* and c-*myc* expression in untreated THP-1 cells. Exposure of THP-1 cells to RA at 1 μmol/L concentration, which causes these cells to adhere, acquire phagocytic functions, and to stop proliferating as determined by ^{3}H-thymidine incorporation,[37] resulted in a high expression of c-*fos* at 48 hours. By 72 hours of treatment, the level of expression had dropped slightly. The level of c-*myb* in RA-treated

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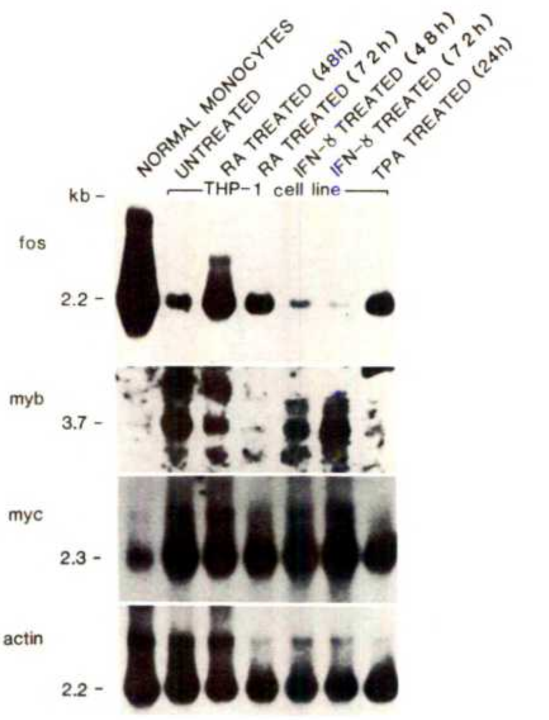

Fig 1. **Northern blot analysis demonstrating c-*fos*, c-*myb*, and c-*myc* expression in HPBM; uninduced THP-1 cells; THP-1 cells treated with 1 μmol/L RA for 48 hours and for 72 hours and THP-1 cells treated with R-IFN-γ (200 units/mL) for 48 hours and for 72 hours; and THP-1 cells treated with TPA (50 ng/mL) for 24 hours.**

THP-1 cells continuously decreased with time and became undetectable by 72 hours of treatment. C-*myc* expression was not affected after 48 and 72 hours of treatment with RA. R-IFN-γ, which did not induce morphological or functional differentiation in THP-1 cells but had a transient antiproliferative effect,[37] caused a slight decrease in c-*fos* expression and no noticeable change in c-*myb* expression even after prolonged exposure (72 hours). Treatment with R-IFN-γ did not affect the expression of c-*myc* in THP-1 cells. Twenty-four–hour exposure to TPA, a potent inducer of morphological and functional differentiation in THP-1 cells, resulted in a marked induction of c-*fos* expression. This was associated with a complete suppression of c-*myb* expression after 24 hours of treatment but with no significant change in the level of c-*myc* expression. As an internal control, the northern blots were rehybridized with a β-actin probe to ensure that the differences observed in the levels of proto-oncogene expression were not attributable to loading unequal amounts of RNA onto the lanes.

To quantitate the levels of expression, we serially diluted total cellular RNA from each sample, dot-blotted it onto nitrocellulose, and hybridized it with the c-*fos*, c-*myb*, and c-*myc* probes (Fig 2). We noted a 32-fold higher c-*fos* expression in HPBM when compared to untreated THP-1 cells; conversely, expression of c-*myb* was 32-fold higher and that of c-*myc* was 16-fold higher in untreated THP-1 cells when compared to the expression in HPBM. RA treatment of THP-1 cells was associated with a 16-fold increase in c-*fos* expression by 48 hours and an eight-fold suppression of c-*myb* expression after 72 hours of treatment when compared with that of untreated cells. THP-1 cells treated with R-IFN-γ showed a slight decrease in the level of c-*fos* expression and no significant change in c-*myb* expression. Treatment of THP-1 cells with TPA resulted in more than an eightfold increase in c-*fos* expression, whereas c-*myb* expression was almost completely abrogated under similar conditions. The increase in c-*fos* expression, induced by RA or TPA in THP-1 cells, was four- to eightfold lower than the levels detected in normal, freshly isolated HPBM and preceded the down regulation of c-*myb* expression. The down regulation in c-*myb* expression was more rapid and more prominent in TPA-treated THP-1 cells than in RA-treated cells. We reprobed the dot blot filters with β-actin and confirmed that changes in oncogene expression were not due to uneven RNA loading (data not shown).

DISCUSSION

The results show that the terminal differentiation of the THP-1 cell line is associated with a "switch" in c-*fos*/c-*myb* expression. At the time of terminal differentiation of THP-1, the levels of c-*fos* and c-*myb* expression approximate the levels detected in HPBM. It is important to point out that the HPBM used in this study were functionally unaltered[28] and were obtained under strict endotoxin-free conditions that represent a "resting state." Once collected, they were immediately processed for analysis and, hence, represent their physiologic state in peripheral blood. The THP-1 cell line has the advantage of being 100% monocytic, as documented by morphologic, biochemical, and enzymatic characterization.[28,29] Similar results were reported from studies of a human promyelocytic leukemia cell line, HL-60.[8,9] These authors showed that differentiation of HL-60 cells toward

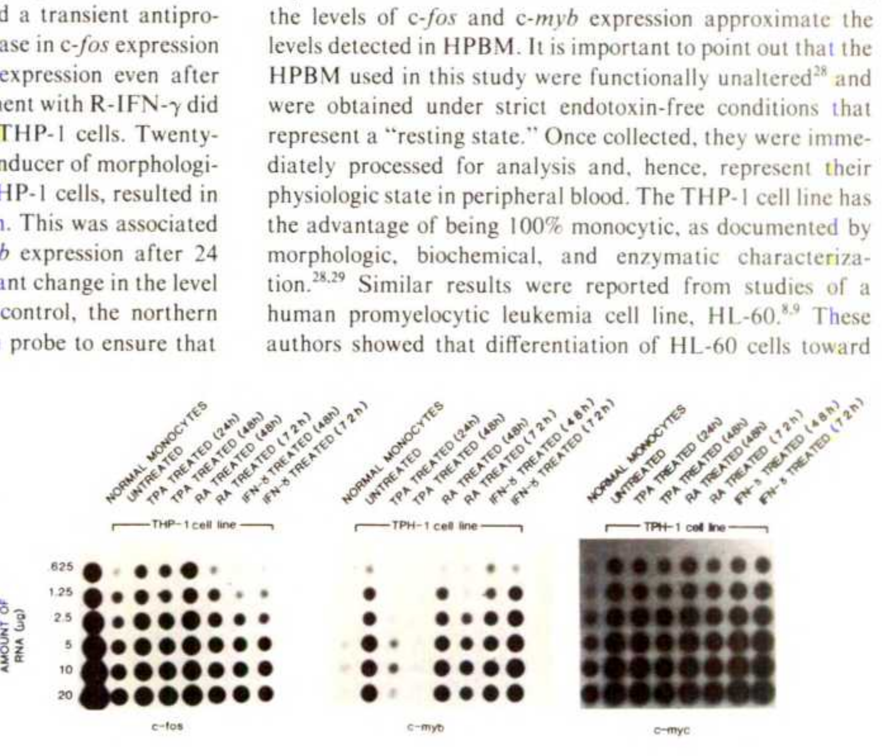

Fig 2. **Dot blot analysis demonstrating quantitative changes in the level of c-*fos*, c-*myb*, and c-*myc* expression in HPBM; untreated THP-1 cells; and THP-1 cells treated with TPA (50 ng/mL), RA (1 μmol/L), or r-IFN-γ (200 units/mL). Total RNA was prepared as described in Fig 1, serially diluted in a solution containing 12.8% formaldehyde and "special" SSC (12 × NaCl and 8 × Na citrate), and dot-blotted onto nitrocellulose. Hybridization was carried out as described in Fig 1.**

the monocytic pathway is accompanied by an enhanced expression of c-*fos* and a decreased expression of c-*myb*. HL-60 cells, which have the potential to differentiate into either granulocytes or monocytes, depending on the inducing signal,[13,14,38] express high levels of c-*fos* only when induced by TPA toward the monocytic pathway. No c-*fos* expression is observed in HL-60 cells when they are induced to differentiate toward the granulocytic pathway.[9] More recently these authors reported that in a TPA-resistant cell variant of the promyelocytic cell line HL-60, expression of c-*fos* is not required for differentiation into macrophages.[39] Thus, no c-*fos* expression was observed when variants HL-525 or HL-534 were induced to differentiate into macrophages with $1.25\ (OH)_2D_3$. The $1.25\ (OH)_2D_3$-induced differentiation, however, was not accompanied by growth arrest, suggesting that the c-*fos* gene product may serve as a signal for growth arrest. In our studies, terminal differentiation and growth arrest were associated with enhanced c-*fos* expression, suggesting that the c-*fos* gene product may serve as a signal to growth arrest during its differentiation toward the monocytic pathway.

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CORRESPONDENCE

GLYCOPROTEIN IIb-IIIa AND PLATELET ADHESION

To the Editor:

The excellent report by Weiss and associates[1] explores the possible role of glycoprotein IIb-IIIa (GPIIb-IIIa), as well as platelet-dense granule substances such as ADP in mediating platelet adhesion. The authors' reference to the work by Timmons and colleagues[2] raises several issues, worth considering further. Timmons and colleagues suggest that the binding of von Willebrand factor (vWF) to GP IIb-IIIa, perhaps mediated by ADP released from platelet-dense granules, may also play a role in mediating platelet adhesion. If ADP mediates binding of vWF to GPIIb-IIIa, enhancing the previous union to subendothelium through GPIb and vWF [consistent with the findings in patients with storage pool deficiency (SPD), thrombasthenia, and Bernard-Soulier syndrome,[1]] the question arises as to how this model works. The ADP source is more likely to be the dense granule content of platelets rather than a contribution from RBCs. The platelet adhesion influenced by the hematocrit values in SPD patients needs further investigation because the number of patients studied was small.

I would like to recall an old subject concerning von Willebrand's disease (vWd) that might be useful in clarifying the conclusions by Timmons and colleagues. Platelet aggregation in vWd is believed to be normal[3]; however, exceptions were found.[4] This intriguing issue led us to study 140 type I von Willebrand patients with differing disease severity.[5] Platelet-induced aggregation with ADP, adrenalin (ADR), collagen (COL), arachidonic acid (AA), and ristocetin (RIPA) was performed in all patients; 47.1% had abnormalities in ADP second-wave aggregation alone or combined with abnormalities in ADR second wave. RIPA was abnormal in only 8.5% of the cases. Aggregation with COL and AA was normal in all patients. In patients with ADP abnormalities, statistical analysis showed a positive correlation with bleeding time and platelet adhesiveness values. The aforementioned data are also consistent with the recent conclusion of Timmons and colleagues,[2] and ADP release reaction in these patients is under current investigation as is possible correlation with hematocrit values. Whether these abnormalities in platelet function represent intrinsically defective von Willebrand platelets remains to be clarified. It seems clear though that ADP, whether contained within platelets or exogenously contributed, plays an important role in platelet adhesion.

Finally, the interaction of fibrinogen (F), vWF, and GPIIb-IIIa is not clearly understood. Competition between vWF and F has been reported. Gralnick and co-workers[6] demonstrated that F concentrations of ~5% of that present in normal plasma still inhibited binding of GPIIb-IIIa and vWF. Others suggest that this should not occur.[7] Thus, it is interesting that the patient with marked deficiency of fibrinogen reported by Weiss and colleagues[1] had normal adhesion with C+S values comparable to those of the normal at a shear rate of 2,600 s^{-1}, particularly because fibrinogen-dependent platelet responses have been reported to be abnormal in such patients. A question then arises: Is fibrinogen so important in the interaction between GPIIb-IIIa and vWF? There may be a preferred interaction of platelets with vWF contained within the platelet or immobilized on a surface.[6] Thus, I think it would be of benefit for those who have the techniques to study the possible role of a propolypeptide of vWF identical to vWAg II[8] in mediating the exposure of GPIIb-IIIa complex to fibrinogen.

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To the Editor:

Dr Harrington comments on our findings in storage pool deficiency which suggest that ADP from platelets (or, possibly, from RBCs under some circumstances) might mediate platelet adhesion.[1] He notes also that he found impaired ADP-induced platelet aggregation in some patients with von Willebrand's disease (vWd) and suggests that this may be evidence that ADP plays a role in von Willebrand factor (vWF)-mediated platelet responses, such as platelet adhesion.[2] We agree that this possibility should be explored further but wish to emphasize the need to distinguish how ADP–

GPIIb-IIIa-vWF might mediate platelet aggregation from the possible role of these factors in mediating platelet adhesion. For example, vWF, bound to GPIIb-IIIa,[3] may support platelet aggregation, particularly when the fibrinogen concentration in the suspension fluid[4] or plasma[5] is very low. In addition, vWF influences platelet-to-platelet interaction (thrombus dimensions) on the subendothelium[6] and in packed bead columns[7] even in the presence of a normal concentration of plasma fibrinogen. The above evidence suggests that, under some circumstances, platelet-to-platelet interactions may be mediated by ADP through mechanisms involving vWF and GPIIb-IIIa. Our studies in storage pool deficiency and thrombasthenia[1] suggest a role for ADP-GPIIb-IIIa in mediating platelet-to-surface reactions as well, but which of the various adhesive proteins (vWf, fibrinogen, fibronectin, thrombospondin), alone or in combination, are involved in this process remains to be determined.

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ERRATA

In the article by Arriaga et al entitled "Interrelationship Between Mitosis and Endomitosis in Cultures of Human Megakaryocyte Progenitor Cells," which appeared in the February 1987 issue (Volume 69, pp 486-492), a typesetting error was made in the formula on page 487. The correct formula appears below:

$$\text{Megakaryocyte DNA content (i)} = \frac{M_i}{\left[\dfrac{\sum_{j=1}^{n} D_j}{n}\right]} \times 2$$

In some copies of the article by Pittaluga et al entitled "Development of T3/T Cell Receptor Gene Expression in Human Pre-T Neoplasms" which appeared in the April 1987 issue (Volume 69, pp 1062-1067), Figures 2 and 4 did not appear. The correct figures appear below:

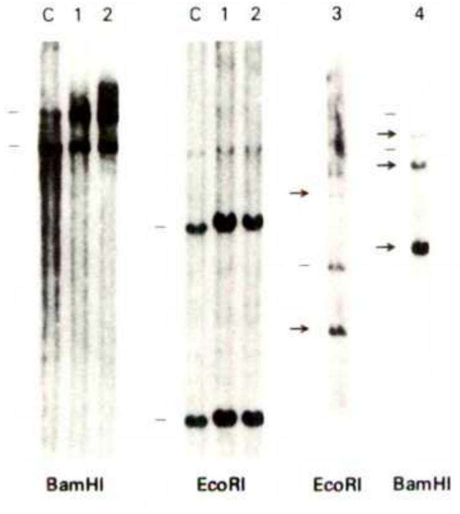

Fig 2. T_γ gene rearrangements. Genomic DNA was digested with the indicated enzyme, size separated in agarose gels, and analyzed on Southern blots with the J_γ probe. Lanes are numbered according to the case number, C represents a germline (unrearranged) control, and the germline bands are indicated with dashes adjacent to the gel. No rearrangements were detected in either case 1 or 2 when digested with both enzymes *Bam*HI or *Eco*RI. Rearrangements were detected in all other cases; for example, two rearrangements were seen in an *Eco*RI digest of case 3, and three rearrangements were present in a *Bam*HI digest of case 4.

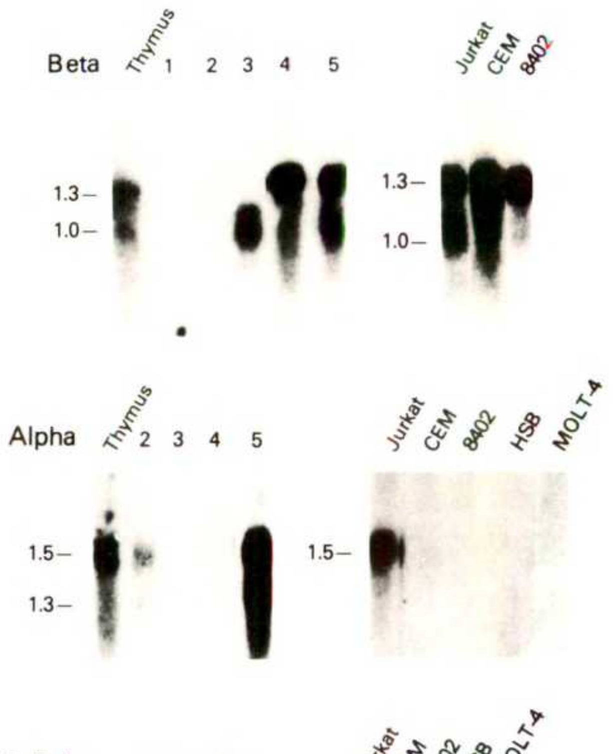

Fig 4. Northern blot analysis of β, α, and T3δ mRNA. Lane numbers refer to the individual case numbers, and mRNA sizes are given in kilobases (kb) on left. β Transcripts were detected in all samples except cases 1 and 2. Both the 1.3-kb and 1.0-kb message sizes could be detected in several cases. T_β transcripts were detected in normal thymus, case 5, and in the Jurkat cell line. No transcripts were seen in either cases 3 or 4, and the extremely faint band in case 2 appearing after prolonged exposure is most likely attributable to a small percentage of normal T cells (~7%) contaminating this sample. T3δ mRNA was found in varying abundancy in most cases but was not detected in cases 1 or 2.

In some copies of the article by Reid et al entitled "Identification of a Functional Role for Human Erythrocyte Sialoglycoproteins β and γ" which appeared in the April 1987 issue (Volume 69, pp 1068-1072), Figure 1 did not appear. The correct figure appears below:

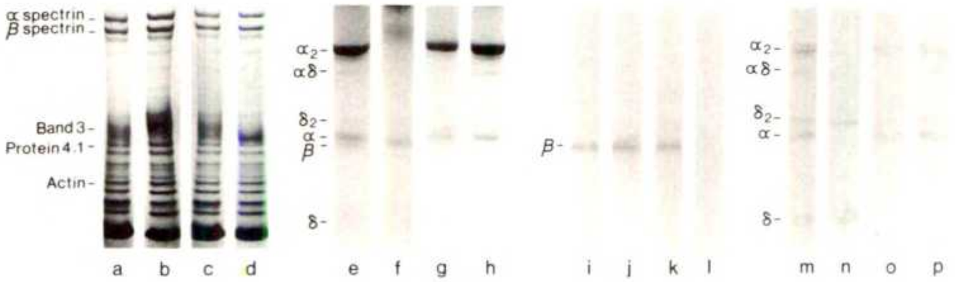

Fig 1. Immunochemical analysis of erythrocyte membranes. Coomassie brilliant blue-stained (lanes a through d), periodic-acid Schiff (PAS)-stained (lanes e through h), and Western blots (lanes i through p) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of sialoglycoprotein (SGP)-deficient membranes: results from a representative example of each type of SGP deficiency. Coomassie brilliant blue staining of SDS-polyacrylamide gradient gels showed that membranes deficient in α (lane b), δ (lane c), or β (lane d) had the normal complement (lane a) of skeletal proteins. The only observed difference between the various samples and control membranes was the decreased mobility of band 3 in the α-deficient membranes. This phenomenon has been described previously and is due to an increase in size of the *N*-glycosidically linked oligosaccharide on band 3.[41] The SGP components of these membrane preparations was determined following staining with PAS (lanes e through h). Membranes from E.P. were deficient in α SGP (lane f), membranes from A.T. were deficient in δ SGP (lane g), and membranes from J.C. were deficient in β SGP (lane h). The PAS-stained gel also illustrates the increased glycosylation of band 3 in the α-deficient membranes. Immunoblotting with antibody BRIC-10 showed that β SGP is absent only in β-deficient membranes (lane l). Normal complement of β SGP is present in control membranes (lane i) and membranes from erythrocytes lacking α or δ SGP (lanes j and k). Immunoblotting with the R1.3 shows that α SGP-deficient erythrocytes lack bands corresponding to monomeric α, dimeric α, and the heterodimer of α and δ (lane n). Membranes from erythrocytes deficient in δ SGP lack monomeric δ, dimeric δ, and the heterodimer of α and δ (lane o). The α and δ SGP content was normal in control erythrocytes (lane m) and in the β-deficient erythrocytes (lane p).

ANNOUNCEMENTS

THE THIRTEENTH INTERNATIONAL SYMPOSIUM FOR COMPARATIVE RESEARCH ON LEUKEMIA AND RELATED DISEASES AND POST-SYMPOSIUM SATELLITE WORKSHOPS

Symposium
November 8-13, 1987
Laromme Hotel, Jerusalem, Israel

Satellite Symposium
November 14-17, 1987
Eliat (Red Sea), Israel

For further information, contact: Dr David S. Yohn, Secretary-General, Suite 302, 410 W Twelfth Ave, Columbus, OH 43210, or Dr Ze'ev Trainin, Kimron Veterinary Institute, PO Box 12, Beit Dagan 50200, Israel.

AN OPEN INTERNATIONAL DISCUSSION SYMPOSIUM "NEWER STRATEGIES IN THE MANAGEMENT OF THROMBOTIC DISORDERS"

November 12-14, 1987
Loyola University Medical Center, Maywood, IL

DISCUSSANTS (PROPOSED)

N. Bang, *Indianapolis, IN*
K. Breddin, *Frankfurt, W Germany*
G.V.R. Born, *London, England*
J.P. Caen, *Paris, France*
J. Caprini, *Chicago, IL*
J. Choay, *Paris, France*
U. Cornelli, *Milan, Italy*
J.F. Davidson, *Glasgow, UK*
J. Fareed, *Chicago, IL*
M. Gent, *Hamilton, Ontario*
V. Gurewich, *Boston, MA*
F. Leja, *Chicago, IL*
V.J. Kakkar, *London, England*
E.F. Mammen, *Detroit, MI*
H.L. Messmore, *Chicago, IL*
J. O'Brien, *Portsmouth, UK*
M. Samama, *Paris, France*
P. Scanlon, *Chicago, IL*
J. Tobelem, *Paris, France*
O.N. Ulutin, *Istanbul, Turkey*
W.H. Wehrmacher, *Chicago, IL*

Topics: heparin and its derivatives; Ticlopidine—newer antiplatelet drugs; T-PA and related thrombolytic agents; Defibrotide and related drugs; viscosity modulators; biotechnology-based products; polytherapy of thrombotic disorders; mechanical devices; physical devices; and target-specific agents.

This is an open symposium and all interested individuals are invited to attend. The program will be available in October and will be forwarded to those interested via circulars. No registration fee is required from the participants; however, the registration is limited to the first 200. *For further information, contact:* Dr J. Fareed, Loyola University Medical Center, 2160 S First Ave, Maywood, IL 60153. Telephone: (312) 531-3256.

THE FIRST INTERNATIONAL SYMPOSIUM ON THE THALASSEMIAS IN CHINA

October 12-15, 1987
Shanghai, PR China

Scientific Organizers: Dr Titus H.J. Huisman, *Augusta, GA*
Dr Clark Lum, *Bethesda, MD*
Dr Zeng Yi-tao, *Shanghai, PR China*

The objective of this conference is to bring together experts from different countries to meet with their Chinese colleagues to stimulate further research in these diseases and to promote and establish scientific contacts.

For further information, contact: Dr Titus H.J. Huisman, Medical College of Georgia, Augusta, GA 30912-3331, or write to the official travel agent: Amerasia Travel Agency, Inc, 3000 Connecticut Ave, NW, Suite 238, Washington, DC 20008.

22ND CONGRESS OF THE INTERNATIONAL SOCIETY OF HEMATOLOGY

August 28–September 2, 1988
Milan, Italy

President: Prof E. Polli
Milan, Italy

For further information please contact the Organizing Secretariat: ISH 88, Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy. Telephone: (02) 702 267 and 783 868. Cable: LORENZFOUND.

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There will be a contributed poster session. Abstracts of 300 words, or less, must relate to one of the six conference subject areas and should indicate the author(s), title of the poster, subject area, and the institution where the research or demonstration project was performed. The deadline for submission of abstracts is **November 1, 1987 and they should be sent to: Dr. Charles F. Whitten, Wayne State University, School of Medicine, 540 East Canfield Avenue, Scott Hall Room 1207, Detroit MI 48201.**

Conference Chairmen:
Charles F. Whitten, M.D., Wayne State University, School of Medicine, Detroit, MI
and
John F. Bertles, M.D., Columbia University College of Physicians & Surgeons, and St. Luke's/Roosevelt Hospital Center, New York City

For program and registration information: Conference Department, The New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021.

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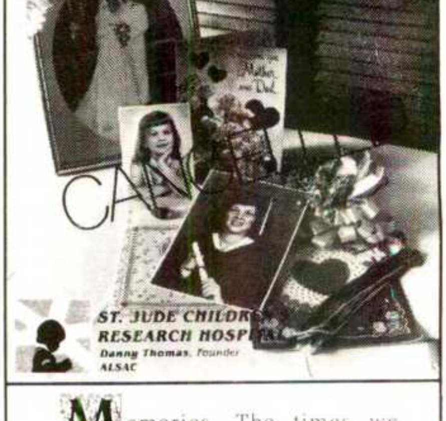
The Unit's work will be closely integrated on one hand with the clinical service, and on the other hand with the already existing laboratory of molecular genetics in the Department of Haematology in which the Unit is located. We are now seeking to appoint a person who will be the scientific leader of the Molecular biology group. The person selected will be offered a career appointment at a level commensurate with his/her scientific record. We are seeking particularly to recruit a scientist with a strong background in any area of molecular or cell biology, who desires to bring previous experience to bear on the understanding of leukaemogenesis and on the pathophysiology of leukaemic cells. Remuneration will be at an appropriate point on the scales for university non-clinical scientific staff. Standard MRC terms of employment apply (an Equal Opportunities Employer). A number of post-doctoral positions will be also available. Interested persons are invited to make contact as soon as possible with the new Director designate, Professor L. Luzzatto, Department of Haematology, RPMS, Ducane Road, London W12 OHS, (telephone 01-740 3234).



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Immune Globulin Intravenous (Human), GAMMAGARD® * is a sterile, dried, highly purified preparation of immunoglobulin which is derived from the cold ethanol fractionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with the appropriate volume of diluent, this preparation contains approximately 50 mg of protein per mL, of which at least 90% is gamma globulin. The reconstituted product contains approximately 1% sodium chloride, not more than 20 mg/mL glucose, not more than 0.2 g/dL PEG, and 0.3M glycine as a stabilizing agent. It has a pH of 6.8±0.4.

The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average, the distribution of IgG subclasses present in this product is the same as is present in normal plasma.[1] Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD contains no preservative.

This product has been prepared from large pools of human plasma which was taken only from plasma units found to have normal levels of alanine aminotransferase (ALT). Each unit of plasma used in the manufacture of this product has been found to be nonreactive for HBsAg and HTLV-III antibody by FDA approved tests.

CLINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum levels is, therefore, to be expected.[2]

As a class, IgG survives longer *in vivo* than other serum proteins.[2,3] Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.[2,3,4] The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.[2,3,4,5]

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.[4,5]

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 μg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea may occasionally occur. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring clinical response and/or the serum IgG levels before each dose, to insure that they do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg per Hr be used. If infusion at this rate causes the patient no distress, the administration rate may be gradually increased but should not exceed 4 mL/kg per Hr.

A rate of administration which is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGARD should be administered as soon after reconstitution as possible. Administration should not begin more than 2 hours after reconstitution.

The reconstituted material should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure the administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGARD is supplied in either 2.5 g or 5.0 g single use vials. Each vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human), GAMMAGARD should be stored under ordinary refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing should be avoided to prevent the diluent bottle from breaking.

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Inquiries:
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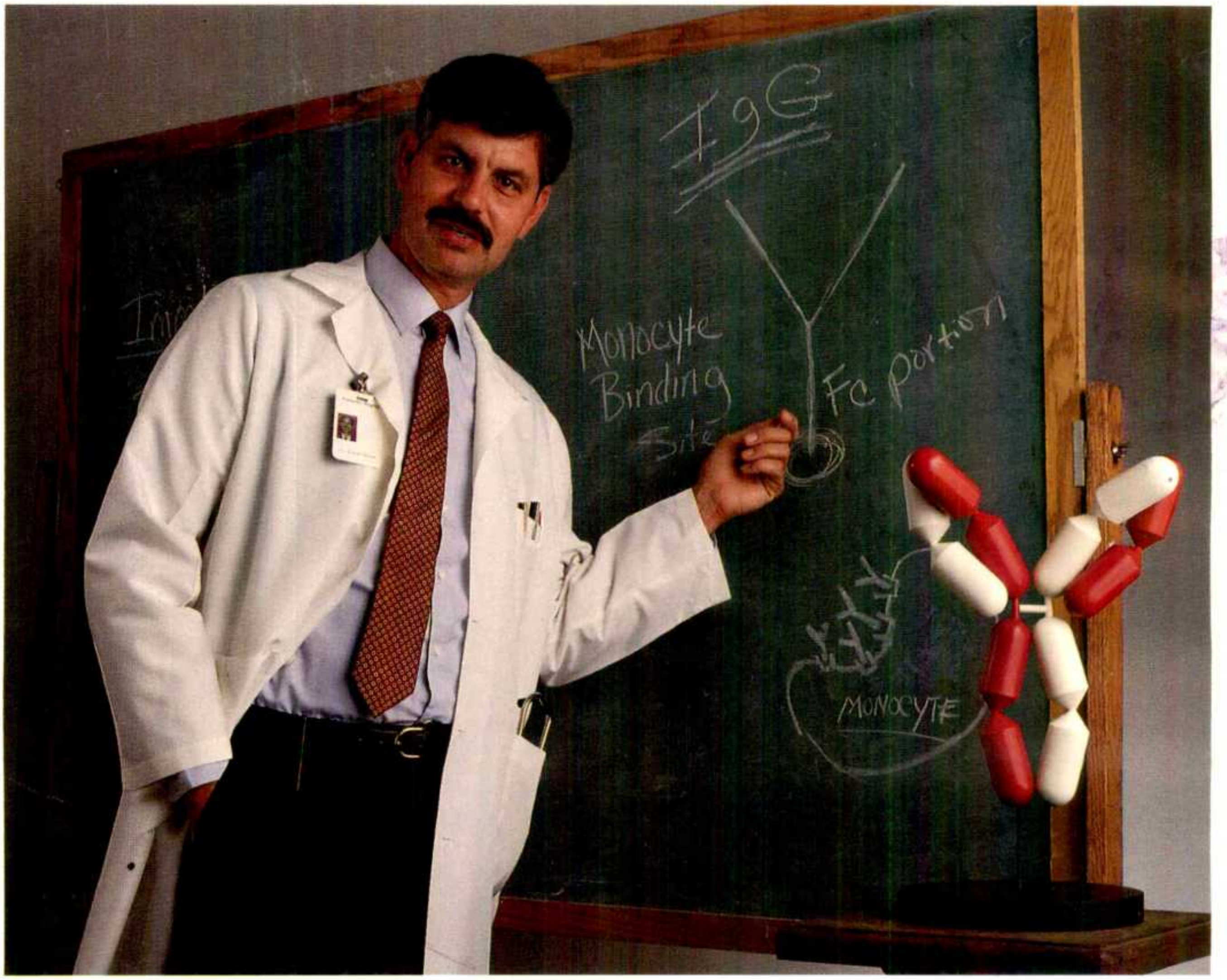

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[1] Jungi, T.W., Santer, M., Lerch, P.G., Barandun, S.: Effect of Various Treatments of Gamma-Globulin (IgG) for Achieving Intravenous Tolerance on the Capacity to Interact with Human Monocyte Fc Receptors. Vox Sang. 51:18-26 (1986).

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[1] Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.

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INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer*, *41*(1):36-51, 1978.
2. Frei E, Blum RH, Pitman SW, *et al:* High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med*, *68*:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro*. *Br J Haematol*, *40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al:* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood*, *55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis*. *135*(3): 493-496, 1977.

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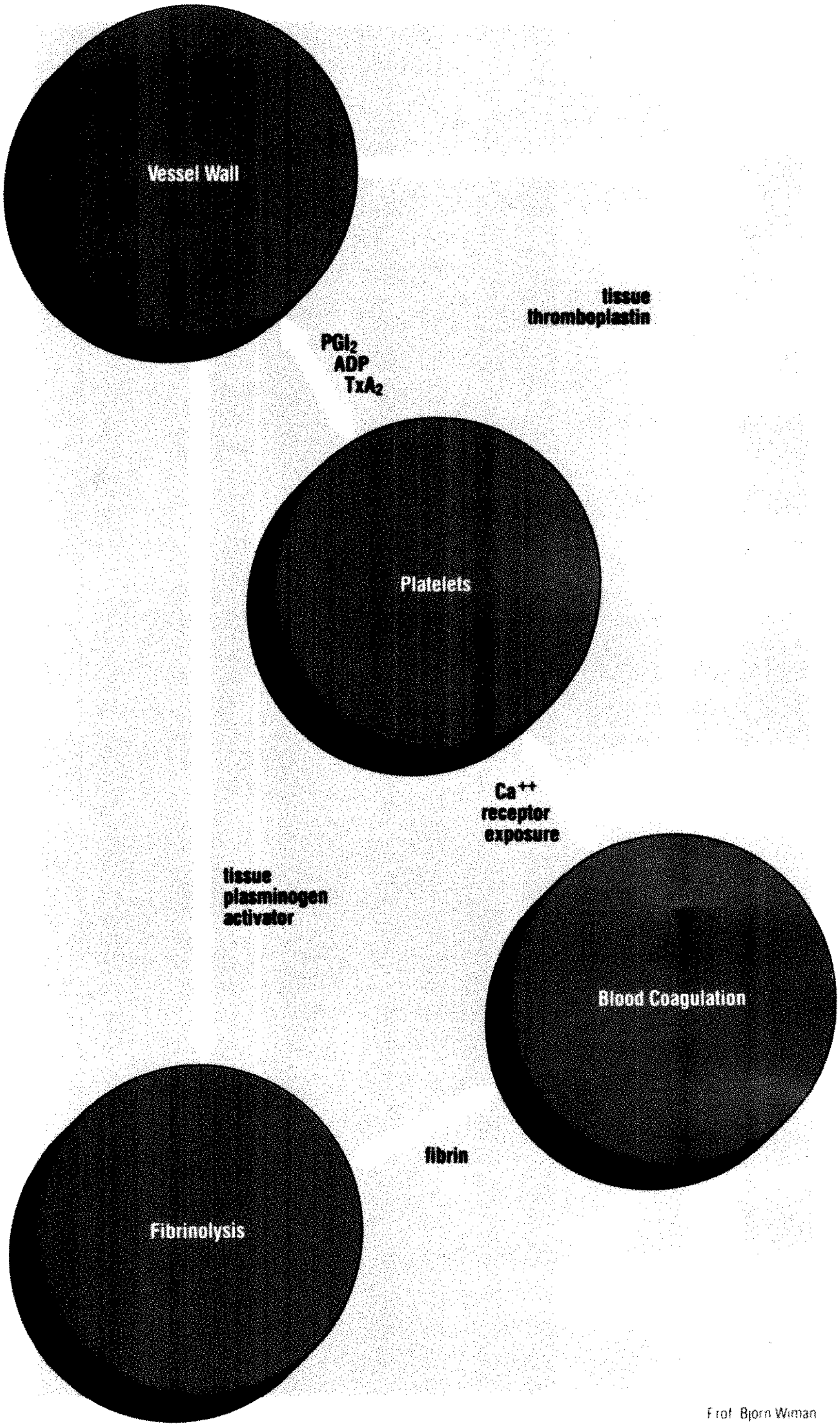

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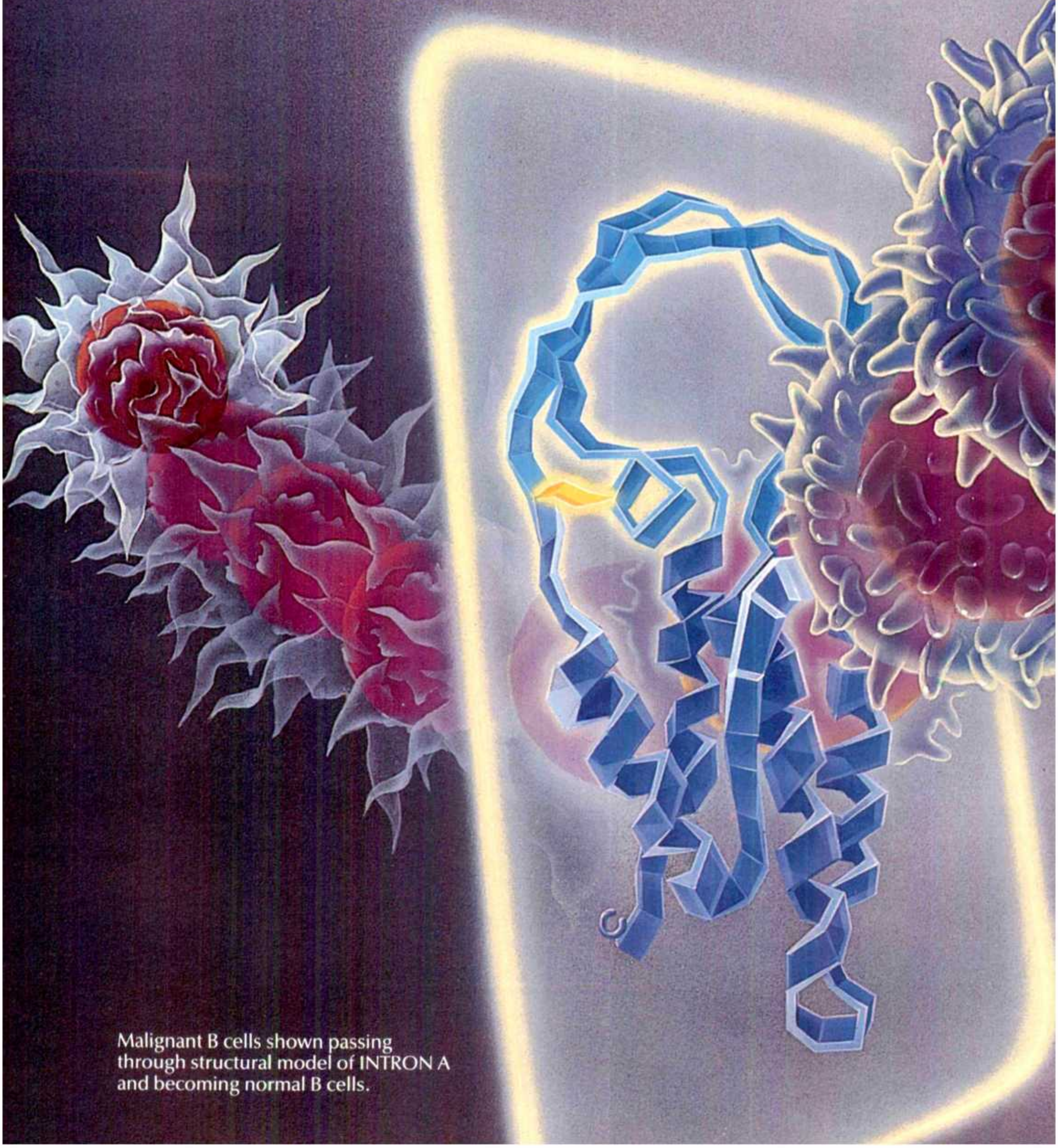

Malignant B cells shown passing through structural model of INTRON A and becoming normal B cells.

EFFICACY WELL ESTABLISHED

The efficacy of INTRON A was established in a 26-center clinical trial involving 145 patients with progressive hairy cell leukemia, a B-cell malignancy.[1-3] INTRON A achieved objective responses in 74% of patients, measured as normalization of granulocyte, hemoglobin, and platelet values and elimination of transfusion dependency.[1]

92% PROBABILITY OF SURVIVAL

In a separate analysis, a 24-month comparison was conducted between the INTRON A study population and historical controls.* After three months of therapy, patients receiving INTRON A reached and maintained a plateau of 92% probability of survival. The probability of survival among historical controls, however, declined steadily to 40% at 24 months.[1]

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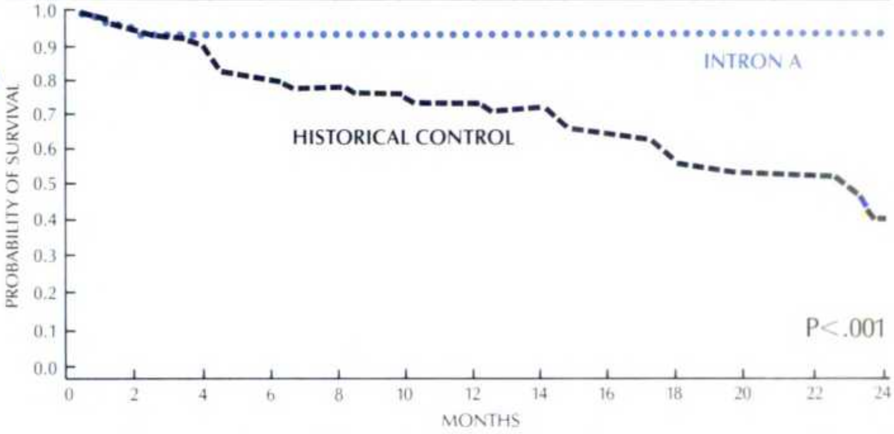

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*Seventy-one historical control patients were treated with current standard therapies, 85% with chemotherapy (mainly chlorambucil) and 15% with supportive care.

DRAMATIC CLINICAL IMPROVEMENTS

More than 80% of the study population achieved normal levels of platelets by three months, of hemoglobin by six months, and of granulocytes by twelve months. Marked decreases in the incidence of life-threatening infections and transfusion dependency preceded normalization of hematologic values.[1]

THERAPY WELL TOLERATED

Treatment-related side effects were generally mild to moderate and rapidly reversible upon discontinuation of therapy. The predominant complaint was a flu-like syndrome, which usually diminished with continued therapy. Mild-to-moderate skin rashes occurred intermittently in 40% of the study population. A low incidence of mild-to-moderate GI disorders was reported.[1]

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BRIEF SUMMARY

INDICATIONS AND USAGE INTRON A for Injection is indicated for the treatment of patients 18 years of age or older with hairy cell leukemia. Studies have shown that INTRON A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and non-splenectomized patients.

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically during treatment to determine whether response to treatment has occurred. If a patient does not respond within 6 months, treatment should be discontinued. If a response to treatment does occur, treatment usually should be continued until no further improvement is observed and these laboratory parameters have been stable for about 3 months (see **DOSAGE AND ADMINISTRATION**). It is not known whether continued treatment after that time point is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS INTRON A is contraindicated in patients with a history of hypersensitivity to interferon alfa or any component of the injection.

WARNINGS Moderate to severe adverse experiences may require modification of the patient's dosage regimen, or in some cases, termination of therapy with INTRON A.

Because of the fever and other "flu-like" symptoms associated with INTRON A administration, it should be used cautiously in patients with debilitating medical conditions, such as those with a history of cardiovascular disease (e.g., unstable angina, uncontrolled congestive heart failure), pulmonary disease (e.g., chronic obstructive pulmonary disease), or diabetes mellitus prone to ketoacidosis. Caution should also be observed in patients with coagulation disorders (e.g., thrombophlebitis, pulmonary embolism) or severe myelosuppression.

Patients with platelet counts of less than 50,000/mm^3 should not be administered INTRON A intramuscularly, but instead by subcutaneous administration.

Cardiovascular adverse experiences which include significant hypotension, arrhythmia, or tachycardia of 150 beats per minute or greater, were observed in approximately 3% of the patients studied who had various malignancies and were treated at doses higher than those for hairy cell leukemia. The incidence of these complications in patients with preexisting heart disease is unknown. Hypotension may occur during administration, or up to two days post therapy, and may require supportive therapy including fluid replacement to maintain intravascular volume. Supraventricular arrhythmias occurred rarely and appeared to be correlated with preexisting conditions, and prior therapy with cardiotoxic agents. These adverse experiences were controlled by modifying the dose or discontinuing treatment, but may require specific additional therapy.

Those patients with a recent history of myocardial infarction and/or previous or current arrhythmic disorder, who require INTRON A therapy, should be closely monitored (see **Laboratory Tests**).

Central nervous system effects manifested by depression, confusion and other alterations of mental status were observed in about 2% of hairy cell leukemia patients treated with INTRON A. The overall incidence in a larger patient population with other malignancies treated with higher doses of INTRON A was 10%. More significant obtundation and coma have been observed in some patients, usually elderly, treated at higher doses for other malignant diseases. These effects are usually rapidly reversible. In a few severe episodes, full resolution of symptoms has taken up to three weeks. Patients should be closely monitored until resolution of these effects. Discontinuation of INTRON A Interferon alfa-2b, recombinant for Injection therapy may be required. Narcotics, hypnotics, or sedatives may be used concurrently with caution.

Acute serious hypersensitivity reactions (e.g., urticaria, angioedema, bronchoconstriction, anaphylaxis, etc.) have not been observed in patients receiving INTRON A; however, if such an acute reaction develops, the drug should be discontinued immediately and appropriate medical therapy instituted. Transient cutaneous rashes have occurred in some patients following injection, but have not necessitated treatment interruption.

Laboratory abnormalities which occurred in hairy cell leukemia patients included elevated SGOT and SGPT, which occurred in 4% and 13% of patients, respectively. The overall incidences of laboratory abnormalities in a larger patient population with other malignancies, and treated at higher doses were somewhat higher. These included elevated liver function tests (SGOT, SGPT in 10% of patients) and reductions in granulocyte (20% of patients) and platelet counts (18% of patients) (see **Laboratory Tests**). These abnormalities are usually mild to moderate and transient. Severe abnormalities of these laboratory parameters are usually rapidly reversible upon cessation or reduction of INTRON A therapy.

PRECAUTIONS Information for Patients Patients being treated with INTRON A should be directed in its appropriate use, informed of benefits and risks associated with treatment and referred to the Patient Information Sheet.

Patients should be cautioned not to change brands of Interferon without medical consultation as a change in dosage may result.

It is advised that patients be well hydrated especially during the initial stages of treatment.

Laboratory Tests In addition to those tests normally required for monitoring patients with hairy cell leukemia, the following laboratory tests are recommended for all patients on INTRON A therapy, prior to beginning treatment and then periodically thereafter:

- Standard hematologic tests — including complete blood counts and differential as well as platelet counts.
- Blood chemistries — electrolytes and liver function tests.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer, should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis, Impairment of Fertility Studies with INTRON A have not been performed to determine carcinogenicity, or the effect on fertility. Interferon may impair fertility. Mutagenicity studies with INTRON A revealed no adverse effects.

Studies in mice, rats, and monkeys injected with INTRON A for up to one month have revealed no evidence of toxicity. However, due to the known species-specificity of interferon, the effects in animals are unlikely to be predictive of those in man.

Pregnancy Category C Animal reproduction studies have not been conducted with INTRON A. It is also not known whether INTRON A can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. INTRON A should be given to a pregnant woman only if clearly needed. Another interferon alfa preparation has been shown to have abortifacient effects in *Macaca mulatta* (rhesus monkeys) when given at 20 to 500 times the human dose. Therefore, INTRON A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. In studies of interferon use in non-human primates, abnormalities of the menstrual cycle have been observed.

INTRON A Interferon alfa-2b, recombinant for Injection should be used with caution in fertile men and women.

Nursing Mothers It is not known whether this drug is excreted in human milk. However, studies in mice have shown that mouse interferons are excreted into the milk. Because of the potential for serious adverse reactions from INTRON A in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use Safety and effectiveness have not been established in patients below the age of 18 years.

ADVERSE REACTIONS Adverse reactions to INTRON A are dose-related. In studies of patients with malignancies other than hairy cell leukemia, hematologic and hepatic toxicities were more common in patients receiving individual injections greater than 10 million IU, than in patients receiving less than this amount. Hematologic, hepatic, cardiovascular and neurologic toxicities were more common in patients receiving more than 30 million IU per injection than in patients receiving less than that amount.

The following adverse reactions were reported to be possibly or probably related to INTRON A therapy during clinical trials. The most frequently reported adverse reactions were flu-like symptoms, primarily fever, fatigue, and chills which occurred in almost all patients treated. Other organ systems in which reactions occurred in more than 5% of the patients are:

General — Taste alteration, anorexia and weight decrease occurred uncommonly.

Cardiovascular — Mild hypotension was frequently recorded although it was rarely symptomatic. Hypertension also occurred less commonly. Tachycardias occurred uncommonly usually in association with high fevers.

Gastrointestinal System — Nausea was common, although vomiting occurred rarely. Mild diarrhea was reported less commonly.

Hematologic — Mild thrombocytopenia and transient granulocytopenia occurred commonly.

Musculo-Skeletal System — Myalgia and arthralgia occurred commonly, usually in association with other flu-like symptoms.

Nervous System — Headache, somnolence, confusion, and dizziness occurred uncommonly. Ataxia and paresthesia were rare.

Psychiatric — Anxiety, depression and nervousness occurred uncommonly.

Skin and Appendages — Mild pruritus occurred uncommonly. Mild alopecia was uncommon. Various transient, mild skin rashes were seen frequently in patients with hairy cell leukemia.

Other Adverse Reactions — The following adverse reactions were reported with an incidence of 5% or less: leg cramps, constipation, insomnia, herpetic eruptions, non-herpetic cold sores, urticaria, hot flashes, supraventricular arrhythmias, epistaxis, stomatitis, paralytic ileus, dehydration, coagulation disorder (elevated PT and PTT), abnormal vision, tremor, emotional lability, chest pain, pharyngitis and syncope.

Adverse reactions reported rarely (less than 1%) were dyspepsia, purpura, dyspnea, sneezing, oculomotor paralysis, nasal congestion, flatulence, increased saliva, hyperglycemia, and ulcerative stomatitis.

Laboratory Value Changes — Those patient laboratory values which were normal to moderately abnormal (WHO grades 0–2) at baseline that worsened to either severe or life-threatening abnormalities (WHO grades 3 or 4) during some phase of treatment included: WBC, platelets, granulocytes, SGOT, creatinine, SGPT, LDH, and alkaline phosphatase.

DOSAGE AND ADMINISTRATION The recommended dosage of INTRON A for Injection for the treatment of hairy cell leukemia is 2 million IU/m^2 administered intramuscularly (see **WARNINGS**) or subcutaneously 3 times a week. Higher doses are not recommended. The normalization of one or more hematologic variables usually begins within 2 months of initiation of therapy. Improvement in all three hematologic variables may require 6 months or more of therapy.

This dosage regimen should be maintained unless the disease progresses rapidly, or severe intolerance is manifested. If severe adverse reactions develop, the dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. If persistent or recurrent intolerance develops following adequate dosage adjustment, or disease progresses, treatment with INTRON A Interferon alfa-2b, recombinant for Injection should be discontinued. The minimum effective dose of INTRON A has not been established.

At the discretion of the physician, the patient may self-administer the dose. The medication may be administered at bedtime.

HOW SUPPLIED INTRON A Interferon alfa-2b, recombinant for Injection is available in 3-, 5-, 10- and 25-million IU vials, each packaged with its own diluent.

Store INTRON A for Injection, both before and after reconstitution, between 2° and 8°C (36° and 46°F). JBS-13762805

References: **1.** Data on file, Schering Corporation. **2.** Jacobs AD, Champlin RE, Golde DW: Recombinant α-2-interferon for hairy cell leukemia. *Blood* 1985; 65:1017-1020. **3.** Golomb HM, Jacobs A, Fefer A, et al: Alpha-2 interferon therapy of hairy-cell leukemia: A multicenter study of 64 patients. *J Clin Oncol* 1986;4:900-905.

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Today's IGIV. Don't settle for less.

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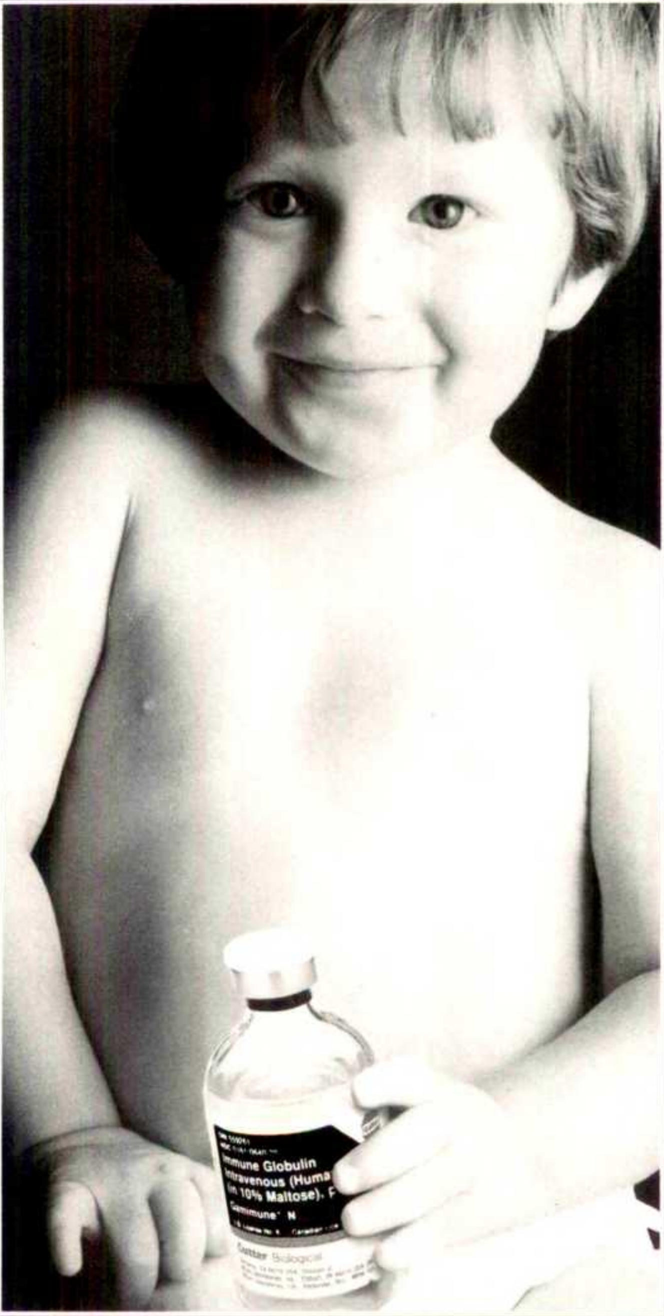
For both chronic and acute ITP in children and adults: rapid elevation of platelet levels

GAMIMUNE® N may initiate a therapeutic increase in platelets in both children and adults with idiopathic thrombocytopenic purpura (ITP).[1][*][†] Treatment with GAMIMUNE® N has enabled some ITP patients to undergo major surgery.[1]



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Cutter's fractionation method (Cohn-Oncley 6 & 9) contains all the necessary steps to produce high purity immune globulin. GAMIMUNE® N contains no foreign contaminants, no detectable levels of protein aggregates or vasoactive enzymes.[2] With GAMIMUNE® N you get what you expect …IGIV containing at least 98% pure gamma globulin.[1]

GAMIMUNE® N

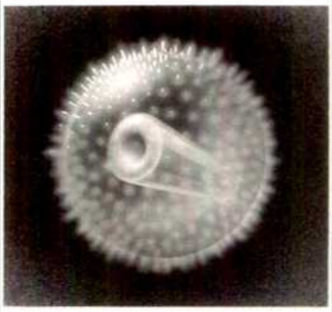
Immune Globulin
Intravenous (Human) 5%
(in 10% Maltose), pH 4.25

"Native" IGIV

GAMIMUNE® N is manufactured without chemical modification. This means GAMIMUNE® N antibodies maintain normal opsonization capacity, have a normal distribution of IgG subgroups and provide the immunologic activity of normal plasma.[1]

Documented in vitro inactivation of HTLV-III virus[‡]

[‡] The Cohn-Oncley process used to produce Gamimune®N has been challenged *in vitro* with a virus spike of 1×10^5 TCID (Tissue Culture Infectious Doses) of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, Gamimune®N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 TCID (Tissue Culture Infectious Doses) reduction of HTLV-III.[3]



Artist's interpretation of HTLV-III virus.

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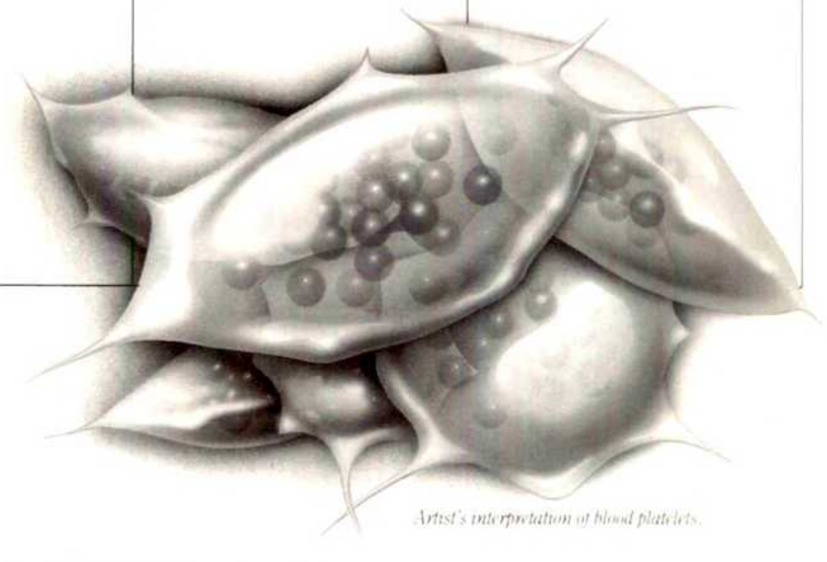
Dose for dose, GAMIMUNE® N saves money by eliminating valuable reconstitution time in the pharmacy. Gram for gram of protein, GAMIMUNE® N is affordable; there is no premium price for TODAY'S IGIV.



Artist's interpretation of blood platelets.

*It is not possible to predict which patients with ITP will respond to therapy.

†In patients who respond to therapy, a rise in platelet count is generally rapid (within 1 to 5 days), transient (most often lasting from several days to several weeks) and should not be considered curative.

From the original IGIV, today's IGIV...it offers more

Please see following page for brief summary of prescribing information.

GAMIMUNE® N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N; at a dose of 400 mg/kg body weight daily for 5 days, there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

U.S. License No. 8

REFERENCES: 1. GAMIMUNE® N Product License Application. **2.** Lundblad JL, Mitra G, Steinberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. Rev Infect Dis 1986;8(Jul–Aug): S382–S390. **3.** Mitra G, Wong MF, Mozen MM, et al: Elimination of infectious retroviruses during preparation of immunoglobulins. Transfusion 1986;26(4):394–397.

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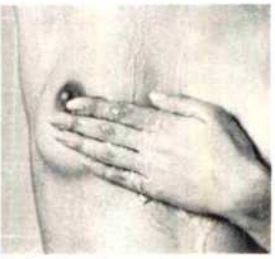

1. In bath or shower.
Fingers flat, move opposite hand gently over each breast. Check for lumps, hard knots, thickening.

2. In front of a mirror.
Observe breasts. Arms at sides. Raise arms high overhead. Any change in nipples, contours, swelling, dimpling of skin? Palms on hips: press down firmly to flex chest muscles. Breasts do not usually match.

3. Lying down.
Pillow under right shoulder, right hand behind head. Left hand fingers flat, press gently in small circular motions starting at 12 o'clock. Make about three circles moving closer to and including nipple. Reverse and repeat on left.

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The journal will publish the description, technical and clinical evaluation of specific molecules and cell lines which may be used in the diagnosis, location and monitoring of disease.

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- Improved methods for the generation or detection of signals from these probes *in vivo* or *in vitro*.
- Animal models used as tests of the probes.

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A high percentage of immunocompromised patients are at risk for developing HSV infections. Timely therapy with an antiviral agent can avoid the incidence of secondary complications, such as visceral dissemination, and prolonged hospital stay.

Appearances may be deceiving

Both slides are herpes simplex. In immunocompromised patients, mucositis and stomatitis that appear to result from radiation therapy or immunosuppression may actually be caused by herpes simplex. Differential diagnosis can be problematic, but appropriate laboratory tests can aid in the determination.

There is an effective therapeutic agent available

ZOVIRAX I.V. stops viral replication, speeds healing, and reduces pain. It is effective antiviral therapy for treatment of herpes simplex viral infections.

ZOVIRAX® I.V.
(acyclovir sodium)

STERILE POWDER / I.V. INFUSION

When the problem begins with a virus, end it with an antiviral.

Please see following page for brief summary of prescribing information.

Both slides are herpes simplex. You may be surprised, but especially in immunocompromised patients it is often difficult to differentiate between herpes infections and mucositis resulting from radiation therapy. Herpes simplex does not always have a classic presentation, and is easily misjudged.

ZOVIRAX® I.V.
(acyclovir sodium)
STERILE POWDER / I.V. INFUSION

ZOVIRAX® I.V. INFUSION (acyclovir sodium) sterile powder

FOR INTRAVENOUS INFUSION ONLY

INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) sterile powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

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EXAMPLES OF REFERENCES

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1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

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8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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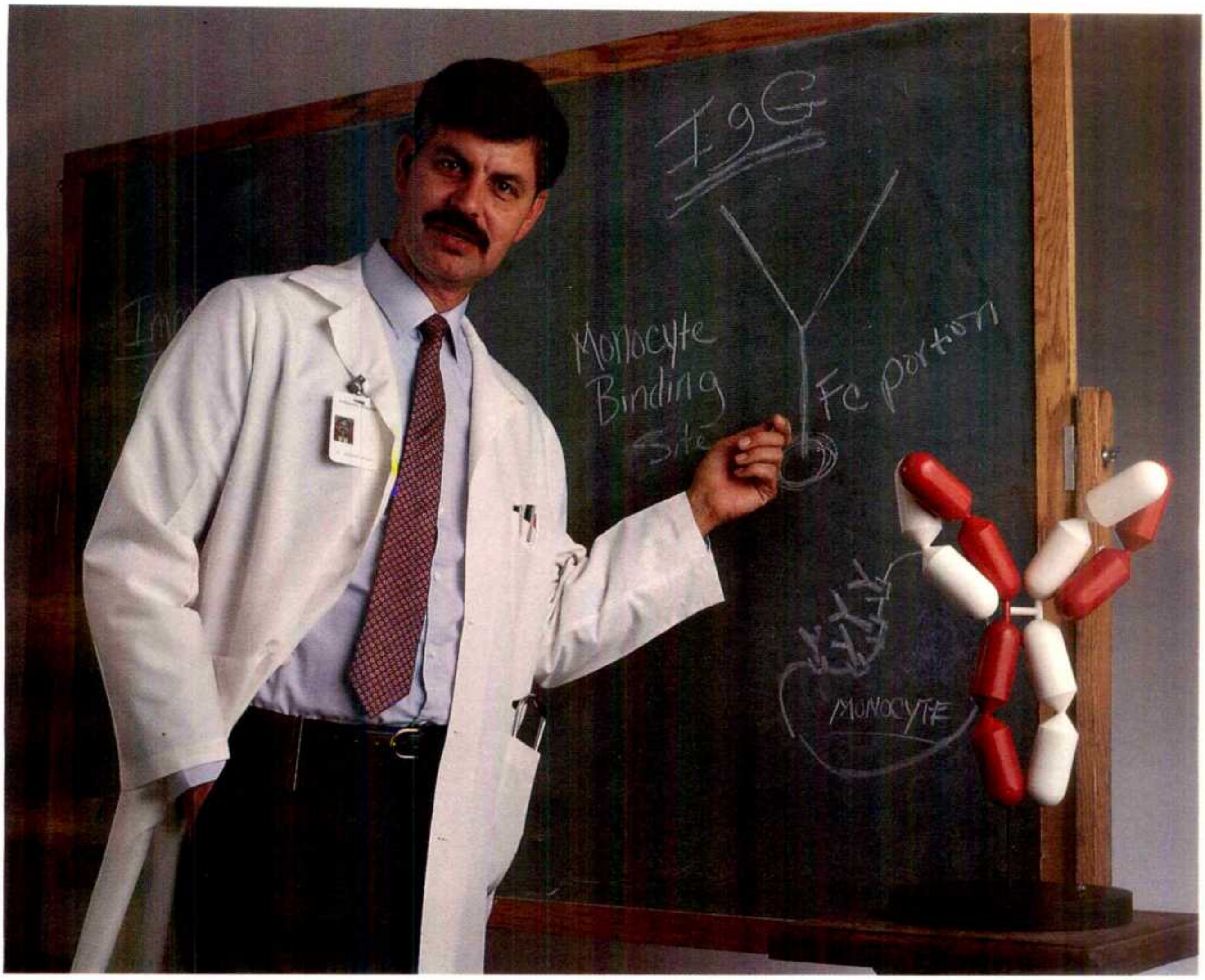

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[1] Jungi, T.W., Santer, M., Lerch, P.G., Barandun, S.: Effect of Various Treatments of Gamma-Globulin (IgG) for Achieving Intravenous Tolerance on the Capacity to Interact with Human Monocyte Fc Receptors. Vox Sang. 51:18-26 (1986).

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The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average, the distribution of IgG subclasses present in this product is the same as is present in normal plasma.[1] Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD contains no preservative.

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Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum levels is, therefore, to be expected.[2]

As a class, IgG survives longer *in vivo* than other serum proteins.[2,3] Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.[2,3,4] The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.[2,3,4,5]

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.[4,5]

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 μg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea may occasionally occur. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring clinical response and/or the serum IgG levels before each dose, to insure that they do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg per Hr be used. If infusion at this rate causes the patient no distress, the administration rate may be gradually increased but should not exceed 4 mL/kg per Hr.

A rate of administration which is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGARD should be administered as soon after reconstitution as possible. Administration should not begin more than 2 hours after reconstitution.

The reconstituted material should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure the administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGARD is supplied in either 2.5 g or 5.0 g single use vials. Each vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human), GAMMAGARD should be stored under ordinary refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing should be avoided to prevent the diluent bottle from breaking.

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Inquiries:
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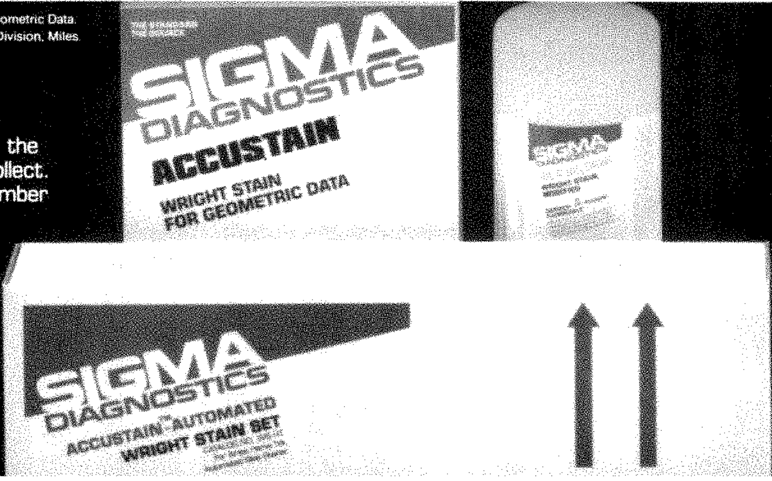
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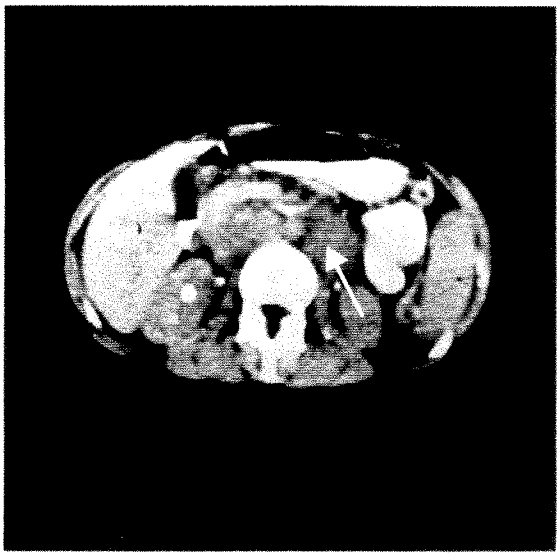
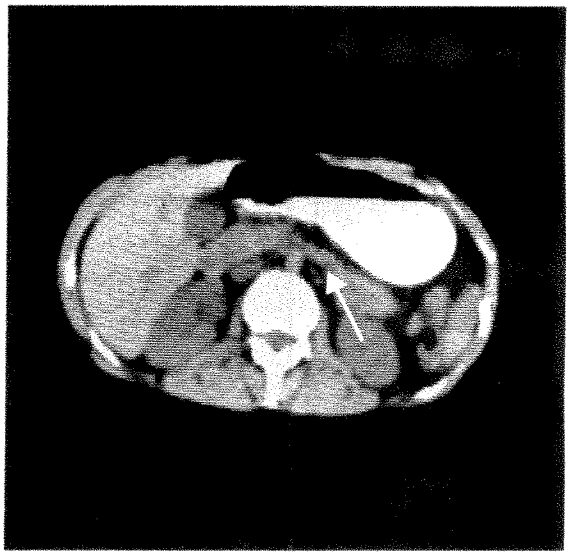


Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
August 4



No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

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BACOP

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COP-BLAM

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*Case information on file at Bristol-Myers Oncology Division

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M-BACOD

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m-BACOD

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MACOP-B

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See prescribing information on following page.

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Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe.

Suggested preventive measures are:

(1) Maintain $FI\ O_2$ at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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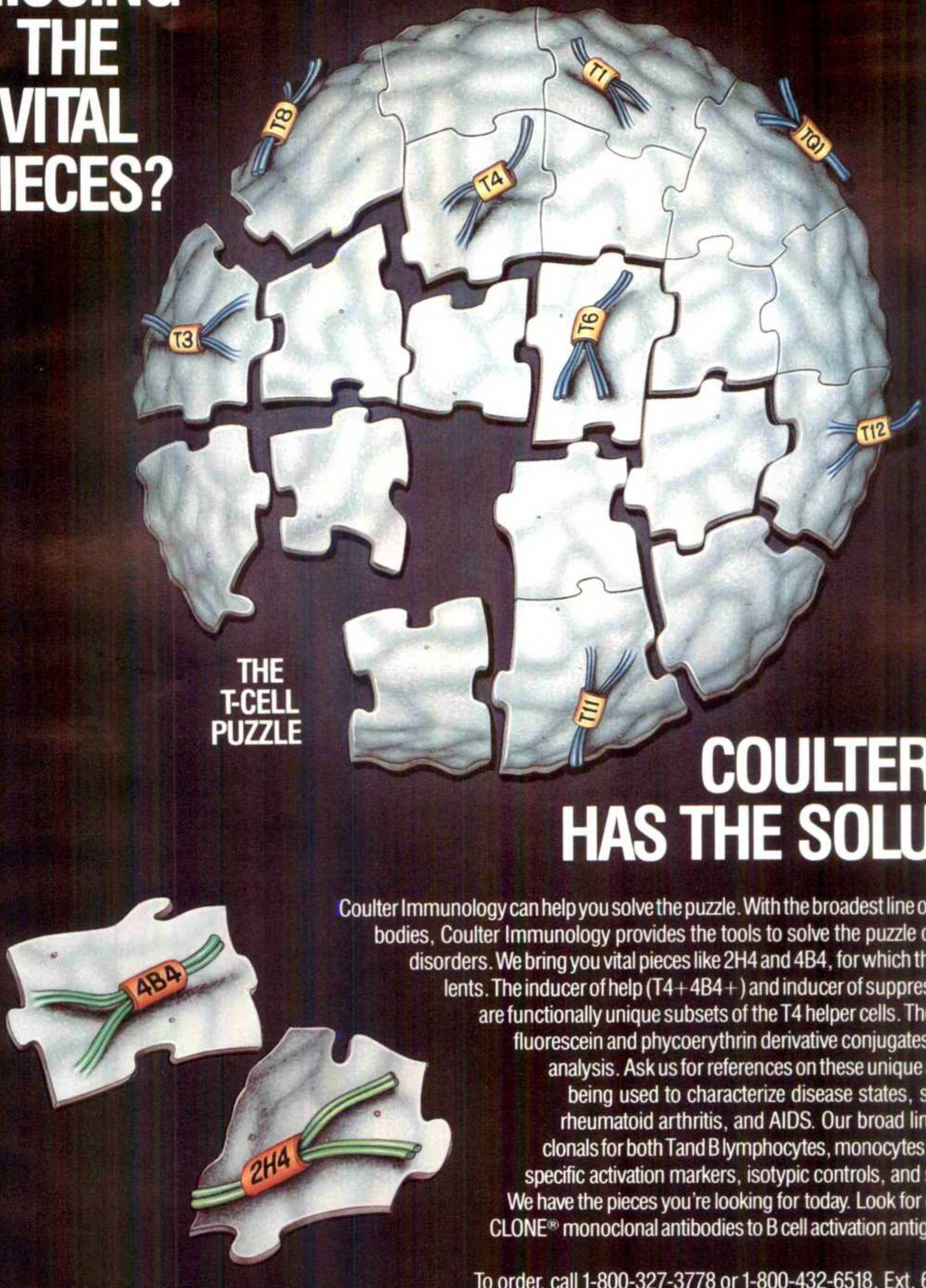

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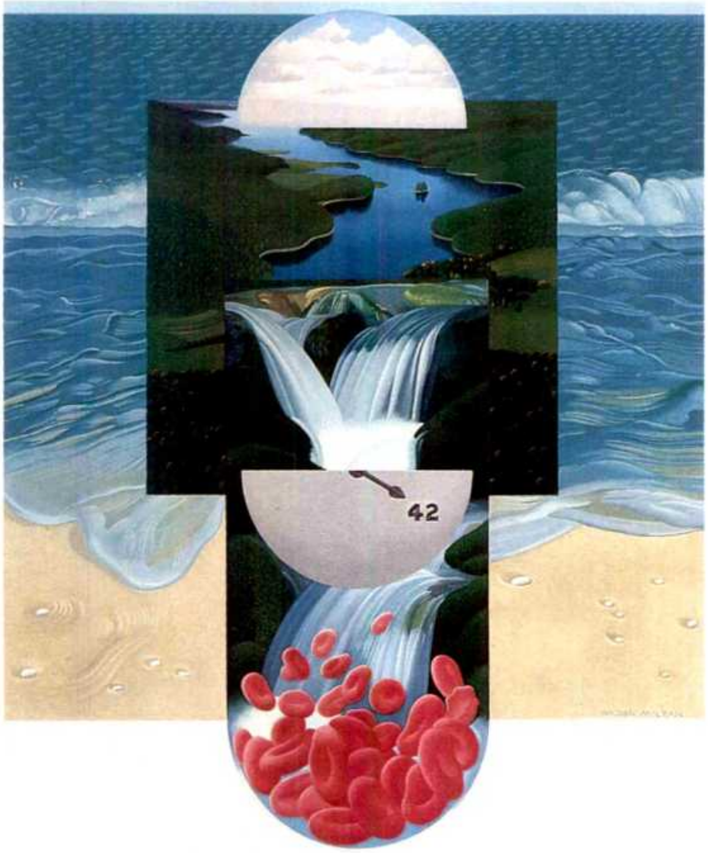

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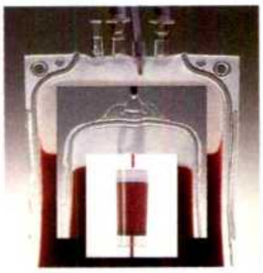

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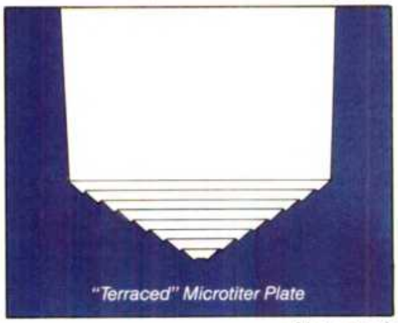
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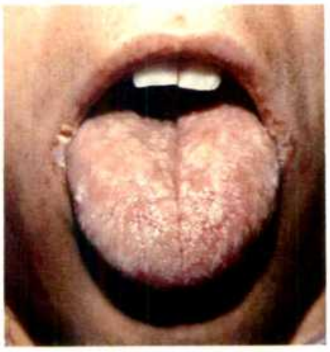
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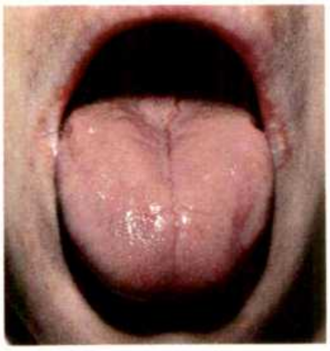
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Based on a study of 52 episodes of oropharyngeal candidiasis, the investigators stated: "We believe that the troche form of clotrimazole...in the treatment of thrush in any patient population...is both effective and safe."[2†]

*Clinical cure is defined as absence of signs and symptoms of the disease.

†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

Mycelex® Troche

(clotrimazole) 10 mg

The only troche for oropharyngeal candidiasis.

MYCELEX® (clotrimazole) TROCHE

Indications and Usage: Mycelex Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications: Mycelex Troches are contraindicated in patients who are hypersensitive to any of the components.

Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C: Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. **2.** Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **3.** Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. **4.** Data on file, Miles Pharmaceuticals.

MILES
Miles Pharmaceuticals
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West Haven, Connecticut 06516 USA

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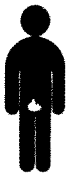
DOM-2242

SIX WARNING SIGNS OF KIDNEY RELATED DISEASES

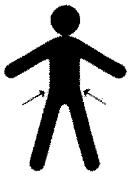


Burning or difficulty during urination

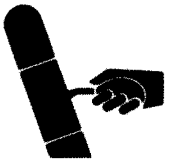
More frequent urination, particularly at night

Passage of bloody-appearing urine

Puffiness around eyes, swelling of hands and feet, especially in children



Pain in small of back just below the ribs (not aggravated by movement)



High Blood Pressure

For further information contact:

KF

National Kidney Foundation of New York, Inc.
432 Park Avenue South
New York, NY 10016

Announcing

RETROVIR® (zidovudine)

The first effective treatment for certain AIDS and other serious HIV* infections

AZT™ (azidothymidine)
IS NOW
RETROVIR® (zidovudine)

*Human immunodeficiency virus

Please see brief summary of prescribing information on last page of this advertisement.

RETROVIR® (zidovudine)

New hope
for many AIDS/ARC patients

A multicenter study proved...

RETROVIR can prolong the lives of certain AIDS and ARC patients

- In a double-blind placebo-controlled clinical trial (N = 281), only one death occurred among 144 RETROVIR patients *v* 19 deaths of the 137 patients on placebo ($p<0.001$). The study population consisted of AIDS patients within 120 days of their first diagnosis of *Pneumocystis carinii* pneumonia or patients with more severe manifestations of AIDS-related complex (ARC)
- The probability of 24-week survival for any patient in the RETROVIR group was 0.98 compared to 0.78 for the placebo group

Comparative Mortality of RETROVIR and Placebo Group Patients in Controlled Clinical Trial (stratified by diagnosis and T4 count) n = 281

Stratum	Group Assigned	Number of Deaths
AIDS (n=160)	**RETROVIR**	**1**
	PLACEBO	12
ARC (n=121)	**RETROVIR**	**0**
	PLACEBO	7

Stratum	Group Assigned	Number of Deaths
Higher T4 count (≥100 ≤500) (n=100)	**RETROVIR**	**0**
	PLACEBO	4
Lower T4 count (<100) (n=181)	**RETROVIR**	**1**
	PLACEBO	15

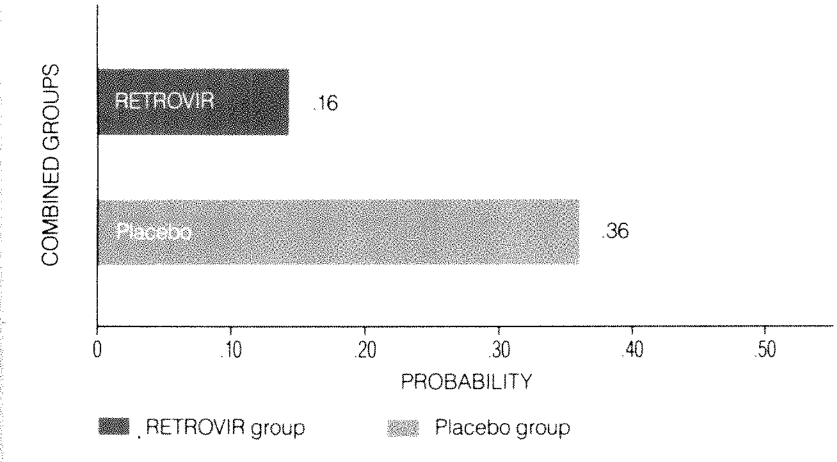
Total Deaths	**RETROVIR**	**1**
	PLACEBO	19

RETROVIR® (zidovudine) reduced the risk and severity of opportunistic infections

- RETROVIR significantly reduced the probability of acquiring opportunistic infections in AIDS and ARC patients ($p<0.001$)
- Severity of opportunistic infections was significantly reduced in RETROVIR patients as compared with controls ($p<0.005$)
- In the controlled trial, no opportunistic infections occurred in ARC patients who had received at least six weeks of RETROVIR therapy. Some ARC patients developed opportunistic infections in the follow-up period while on drug

Probability of Developing Opportunistic Infection Within 24 Weeks

(Excludes opportunistic infections acquired within six weeks of entry into the study)



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WARNING

WARNING: Therapy with RETROVIR® (zidovudine) is often associated with hematologic toxicity including granulocytopenia and severe anemia requiring transfusions (see WARNINGS). In addition, patients treated with zidovudine may continue to develop opportunistic infections (OI's) and other complications of the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) caused by the human immunodeficiency virus (HIV). Therefore, patients on zidovudine should be under close clinical observation by physicians experienced in the treatment of patients with diseases associated with HIV. The safety and efficacy of zidovudine have been established only for certain adult AIDS and advanced ARC patients (see INDICATIONS AND USAGE).

IMPROVING LIVES THROUGH
ANTIVIRAL RESEARCH

BURROUGHS WELLCOME CO.

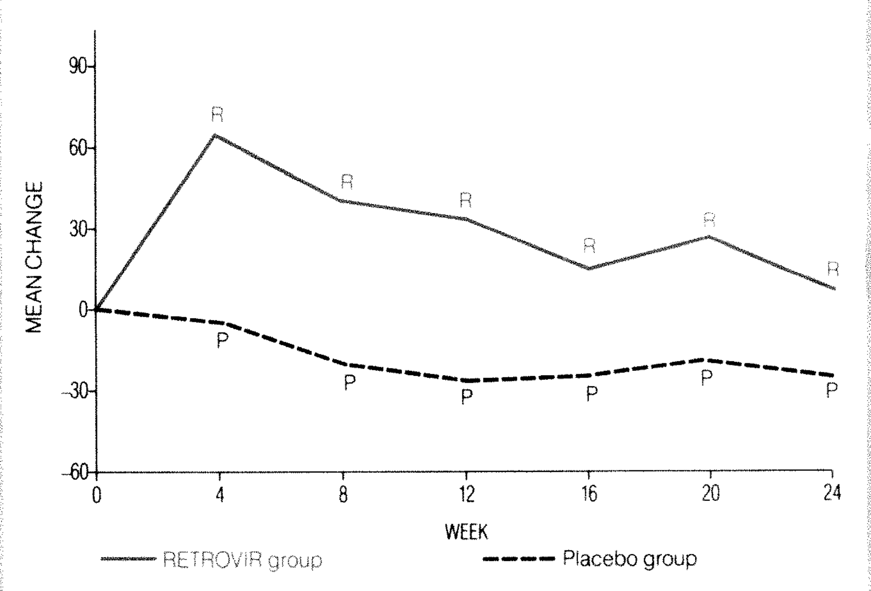
RETROVIR® (zidovudine)

New hope
for many AIDS/ARC patients

RETROVIR improved immune status

- T4 cell counts in the RETROVIR group remained significantly higher than in the control group throughout the study
  - In some RETROVIR patients, particularly those with lower T4 cell counts at entry, T4 cell numbers increased, then subsequently declined
  - In contrast, placebo-group patients experienced a progressive decline from baseline in number of T4 cells
- The number of positive delayed hypersensitivity skin test conversions was significantly higher in the RETROVIR group ($p<0.001$)

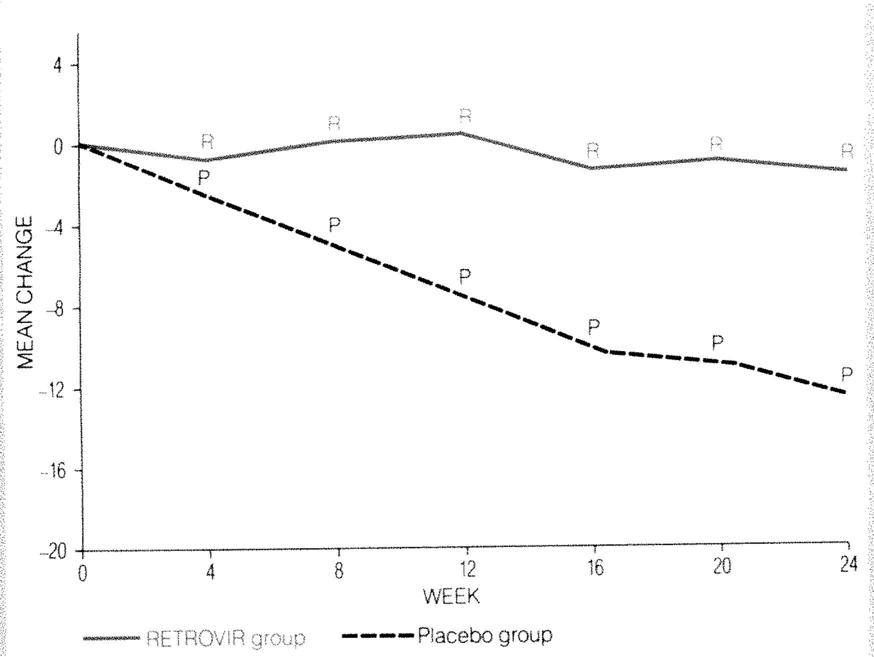
Change in T4 Cell Count



Patients' general health improved

- Patients on RETROVIR® (zidovudine) maintained their high Karnofsky performance scores (an index measuring ability to perform activities of daily life), whereas placebo group patients' scores declined
- RETROVIR patients experienced significantly fewer symptoms related to HIV infection than those on placebo
- Patients on RETROVIR therapy maintained their body weight throughout the study, whereas placebo group patients progressively lost weight

Change in Karnofsky Score for All AIDS/ARC Patients Combined



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IMPROVING LIVES THROUGH
ANTIVIRAL RESEARCH

BURROUGHS WELLCOME CO.

RETROVIR® (zidovudine)

New hope
for many AIDS/ARC patients

Clinical studies showed...

It is important to identify patients at greates risk for bone marrow toxicity, and subsequent RBC and WBC suppression, and monitor carefully

- 25% of RETROVIR patients developed anemia (<7.5 g/dl) as compared to 4% in the placebo group. This adverse reaction was most likely to occur in AIDS patients and patients with lower numbers of T4 cells
- 31% of RETROVIR patients required at least one transfusion, compared with 11% of placebo patients
- Granulocyte depression ($<750/\text{mm}^3$) was observed in 39% of RETROVIR patients as compared with 9% of placebo patients. Granulocytopenia occurred more frequently in AIDS patients and patients with lower T4 cell counts

Clinical adverse events

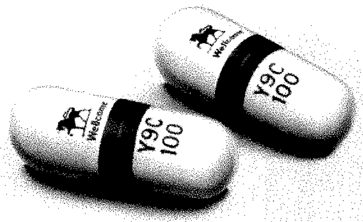
- Although the majority of patients in *both* the RETROVIR (84%) and control groups (72%) reported the occurrence of at least one adverse event, nausea, myalgia, insomnia, and moderate-to-severe headache were significantly more frequent among RETROVIR patients

The potential hematologic toxicity of RETROVIR plays an important role in patient management

- The majority of patients who required adjustment of RETROVIR dosage had hematologic toxicity, specifically anemia or granulocytopenia, usually occurring within the first four to eight weeks of RETROVIR therapy
- Because of this potential, careful monitoring of hematologic indices is recommended every two weeks*

Drug interactions must be considered with RETROVIR

- Concomitant use of acetaminophen with RETROVIR may potentiate granulocytopenia; other drugs that may interfere with RETROVIR metabolism or excretion (eg, probenecid, lorazepam, indomethacin, cimetidine) should be avoided



RETROVIR — the newest antiviral breakthrough from Burroughs Wellcome Co. — is the first effective treatment for certain HIV infections.

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*RETROVIR dose interruption may be required for significant anemia or granulocytopenia until evidence of bone marrow recovery is observed. A reduction in daily dose may be adequate for less severe depression of RBC/WBC counts.

Since the initial supply of RETROVIR® (zidovudine) is limited, a special distribution program is in place

- Rapidity of development and complexity of the production process have limited the current supply of RETROVIR
- To help ensure a continuous supply of RETROVIR for patients at greatest need and those who would benefit most from treatment during this period of limited supply, a special distribution program has been established
- An independent group of experts from the Infectious Disease Society of America (IDSA) has assisted in developing this program for the best utilization of RETROVIR

Patients can be enrolled in the RETROVIR distribution program now

- You can apply to enroll your patients in the RETROVIR distribution program at this time
- If you are currently treating AIDS or ARC patients and need additional information, call 1-800-843-9388
- Detailed information and enrollment applications are also available from your Burroughs Wellcome Co. Sales Representative
- Only patients enrolled in the distribution program will be able to receive RETROVIR

Burroughs Wellcome Co.: Leader in Antiviral Research

- Continuing clinical trials to evaluate further the role of RETROVIR in the treatment of HIV infections
- Conducting research for antiviral agents effective against varicella-zoster, Epstein-Barr, human papilloma, hepatitis B virus, and cytomegalovirus
- Providing physicians with antiviral products effective against herpes simplex — today's hope for patients with genital herpes
- **Pledging to keep you updated as new information becomes available**

If you have patients with HIV infections and need additional information, please call

1-800-843-9388

IMPROVING LIVES THROUGH
ANTIVIRAL RESEARCH

BURROUGHS WELLCOME CO.

Please see brief summary of prescribing information on last page of this advertisement.

RETROVIR® (ZIDOVUDINE) Capsules

WARNING: THERAPY WITH RETROVIR (ZIDOVUDINE) IS OFTEN ASSOCIATED WITH HEMATOLOGIC TOXICITY INCLUDING GRANULOCYTOPENIA AND SEVERE ANEMIA REQUIRING TRANSFUSIONS (SEE WARNINGS).

IN ADDITION, PATIENTS TREATED WITH ZIDOVUDINE MAY CONTINUE TO DEVELOP OPPORTUNISTIC INFECTIONS (OI'S) AND OTHER COMPLICATIONS OF THE ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) AND AIDS RELATED COMPLEX (ARC) CAUSED BY THE HUMAN IMMUNODEFICIENCY VIRUS (HIV). THEREFORE, PATIENTS ON ZIDOVUDINE SHOULD BE UNDER CLOSE CLINICAL OBSERVATION BY PHYSICIANS EXPERIENCED IN THE TREATMENT OF PATIENTS WITH DISEASES ASSOCIATED WITH HIV. THE SAFETY AND EFFICACY OF ZIDOVUDINE HAVE BEEN ESTABLISHED ONLY FOR CERTAIN ADULT AIDS AND ADVANCED ARC PATIENTS (SEE "INDICATIONS AND USAGE").

INDICATIONS AND USAGE: Retrovir Capsules are indicated for the management of certain adult patients with symptomatic HIV infection (AIDS and advanced ARC) who have a history of cytologically confirmed *Pneumocystis carinii* pneumonia (PCP) or an absolute CD4 (T4 helper/inducer) lymphocyte count of less than $200/mm^3$ in the peripheral blood before therapy is begun.

This indication is based primarily on the results of a randomized, double-blind, placebo-controlled trial conducted at 12 medical centers in the United States in which 281 patients with AIDS or advanced ARC were studied for an average of four and a half months. Additional data have been collected on approximately 80% of these patients who have received zidovudine in an open-label extension of this trial for an average of five more months.

The patient population of the controlled trial consisted of 160 AIDS patients (85 Retrovir and 75 placebo) who had recovered from their first episode of PCP diagnosed within the previous four months, and 121 ARC patients (59 Retrovir and 62 placebo) with multiple signs and symptoms of HIV infection, including mucocutaneous candidiasis and/or unexplained weight loss (≥ 15 lbs or > 10% of prior body weight). All patients had evidence of impaired cellular immunity with an absence of delayed cutaneous hypersensitivity and a decreased number of CD4 (T4) lymphocytes in the peripheral circulation. Two hundred twenty-one (79% of all patients) had fewer than 200 T4 $cells/mm^3$ at entry (95% of AIDS patients and 57% of ARC patients). All patients began therapy at a dose of 250 mg every 4 hours around the clock. This dosage was reduced or temporarily or permanently discontinued if serious marrow toxicity occurred. The trial was stopped because of a significant reduction in mortality before all patients had completed the planned 24 weeks of treatment. There were 19 deaths in the placebo group and 1 in the Retrovir group ($p < .001$). All deaths were apparently due to opportunistic infections (OI) or other complications of HIV infection. Treatment duration ranged from 12 weeks to 26 weeks, with a mean and median duration of 17 and 18 weeks, respectively.

Retrovir also significantly reduced the risk of acquiring an AIDS-defining OI in patients after 6 weeks of treatment ($p < .001$). In addition, patients who received Retrovir generally did better than the placebo group in terms of several other measures of efficacy including performance level, neuropsychiatric function, maintenance of body weight and the number and severity of symptoms associated with HIV infection. A modest increase in mean CD4 (T4) counts was seen in the zidovudine group but the significance of this finding is unclear as the CD4 (T4) counts declined again in some patients.

The most significant adverse reaction noted in the study was a depression of formed elements in the peripheral blood, which necessitated dose reduction or drug discontinuation in 49 of the 144 (34%) patients receiving Retrovir. Of those participants whose baseline CD4 (T4) lymphocyte counts were less than 200, 47% of those receiving Retrovir and 10% of those receiving placebo developed a granulocyte count of $<750/mm^3$. Similarly, 45% of Retrovir recipients and only 14% of placebo recipients had a 25% or greater reduction in hemoglobin. (See ADVERSE REACTIONS.)

At the conclusion of the placebo-controlled trial, 127 Retrovir recipients and 100 placebo recipients elected to enroll in an uncontrolled extension protocol in which all patients received Retrovir at a dose of 200 mg every four hours. This dose was chosen because of concern about cumulative hematologic toxicity and to allow for greater flexibility in dosing. Over the subsequent five months, opportunistic infections continued to occur and additional patients died in both groups. Sixteen patients from the original placebo cohort died after the conclusion of the controlled trial. Four of these 16 never received Retrovir and seven expired during the first month of therapy. Ten additional deaths occurred among the original Retrovir recipients. With respect to these data, termination of the controlled trial precludes direct comparisons of mortality and morbidity between the original placebo and drug recipients. Long-term follow-up of these patients, however, will allow for examination of the natural history of prolonged zidovudine therapy.

CONTRAINDICATIONS: Retrovir Capsules are contraindicated for patients who have potentially life-threatening allergic reactions to any of the components of the formulation.

WARNINGS: Zidovudine has been carefully studied in limited numbers of seriously ill HIV-infected patients treated for a limited period of time. Therefore, the full safety and efficacy profile of zidovudine has not been completely defined, particularly in regard to prolonged use, and especially in HIV-infected individuals who have less advanced disease.

Zidovudine should be used with extreme caution in patients who have bone marrow compromise evidenced by granulocyte count $< 1000/mm^3$ or hemoglobin < 9.5 g/dl. In the placebo-controlled study, anemia and granulocytopenia were the most significant adverse events observed (see ADVERSE REACTIONS).

Significant anemia most commonly occurred after 4 to 6 weeks of therapy and in many cases required dose adjustment, discontinuation of zidovudine, and/or blood transfusions. Frequent (at least every 2 weeks) blood counts are strongly recommended in patients taking zidovudine. If anemia or granulocytopenia develops, dosage adjustments may be necessary (see DOSAGE AND ADMINISTRATION).

Coadministration of zidovudine with other drugs metabolized by glucuronidation should be avoided because the toxicity of either drug may be potentiated (see Drug Interactions under PRECAUTIONS). Zidovudine recipients who used acetaminophen during the controlled trial had an increased incidence of granulocytopenia which appeared to be correlated with the duration of acetaminophen use.

PRECAUTIONS:

General: Zidovudine is eliminated from the body primarily by renal excretion following metabolism in the liver (glucuronidation). There are currently no data available concerning the use of zidovudine in patients with impaired renal or hepatic function, and such patients may be at a greater risk of toxicity from zidovudine.

Drug Interactions: The interaction of other drugs with zidovudine has not been studied in a systematic manner. Coadministration of zidovudine with drugs that are nephrotoxic, cytotoxic, or which interfere with RBC/WBC number or function (e.g., dapsone, pentamidine, amphotericin B, flucytosine, vincristine, vinblastine, adriamycin, or interferon) may increase the risk of toxicity. Limited data suggest that probenecid may inhibit glucuronidation and/or reduce renal excretion of zidovudine. In addition, other drugs (e.g., acetaminophen, aspirin, or indomethacin) may competitively inhibit glucuronidation (see WARNINGS).

Some experimental nucleoside analogues which are being evaluated in AIDS and ARC patients may affect RBC/WBC number or function and may increase the potential for hematologic toxicity of zidovudine. Some experimental nucleoside analogues affecting DNA replication antagonize the *in vitro* antiviral activity of zidovudine against HIV and thus, concomitant use of such drugs should be avoided.

Some drugs such as trimethoprim-sulfamethoxazole, pyrimethamine, and acyclovir may be necessary for the management or prevention of opportunistic infections. In the controlled trial, increased toxicity was not detected with limited exposure to these drugs. However, there are two published reports of neurotoxicity (one of profound lethargy and one of seizure) associated with concomitant use of zidovudine and acyclovir.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Long-term carcinogenicity studies of zidovudine in animals have not been completed. However, in an *in vitro* mammalian cell transformation assay, zidovudine was positive at concentrations of 0.5 μg/ml and higher.

No evidence of mutagenicity (with or without metabolic activation) was observed in the Ames *Salmonella* mutagenicity assay. In a mutagenicity assay conducted in $L5178Y/TK^{+/-}$ mouse lymphoma cells, zidovudine was weakly mutagenic in the absence of metabolic activation only at the highest concentrations tested (4000 and 5000 μg/ml). In the presence of metabolic activation, the drug was weakly mutagenic at concentrations of 1000 μg/ml and higher. In an *in vitro* cytogenetic study performed in cultured human lymphocytes, zidovudine induced dose-related structural chromosomal abnormalities at concentrations of 3 μg/ml and higher. No such effects were noted at the two lowest concentrations tested, 0.3 and 1 μg/ml.

Pregnancy: Pregnancy Category C. An oral teratology study in pregnant rats using doses up to 20 times the human dose has revealed no evidence of harm to the fetus due to zidovudine. It is not known whether zidovudine can cause fetal harm when administered to a pregnant woman or can affect reproductive capacity. Zidovudine should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether zidovudine is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions from zidovudine in nursing infants, mothers should be instructed to discontinue nursing if they are receiving zidovudine.

Pediatric Use: Safety and effectiveness in children have not been established.

ADVERSE REACTIONS: The most frequent adverse events and abnormal laboratory values reported in the placebo-controlled clinical trial of oral zidovudine administration in 281 patients (144 patients zidovudine; 137 patients placebo) were granulocytopenia and anemia. The occurrence of these hematologic toxicities was inversely related to CD4 (T4) lymphocyte number, hemoglobin, and granulocyte count at study entry, and directly releated to dose and duration of therapy. The frequency of granulocytopenia and anemia according to the patients' CD4 (T4) levels is shown in the following table:

	Pretreatment CD4 (T4) Levels			
	$\leq 200/mm^3$		$>200/mm^3$	
Abnormality	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia ($< 750/mm^3$)	47%	10%	10%	3%
Anemia (Hgb <7.5 g/dl)	30%	6%	3%	0%

Because many patients were anemic and/or granulocytopenic before starting therapy with zidovudine, examining the degree of change when compared to baseline, as shown in the table below, may be more informative.

		Pretreatment CD4 (T4) Levels			
		$\leq 200/mm^3$		$>200/mm^3$	
Abnormality	% Decrease from Baseline	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia	>50%	55%	19%	40%	13%
Anemia	>25%	45%	14%	10%	10%

The anemia appeared to be the result of impaired erythrocyte maturation as evidenced by increasing macrocytosis (MCV) while on drug.

The 281 patients treated in this controlled trial had serious underlying disease with multiple baseline symptoms and clinical abnormalities. The following table summarizes those reported clinical adverse events which occurred in at least 5% of all patients treated with zidovudine. Severe headache, nausea, insomnia and myalgia were reported at a significantly greater rate in zidovudine recipients.

Percentage (%) of Patients with Clinical Events					
Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %	Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %
BODY AS A WHOLE			MUSCULOSKELETAL		
Asthenia	19	18	Myalgia	8	2
Diaphoresis	5	4	NERVOUS		
Fever	16	12	Dizziness	6	4
Headache	42	37	Insomnia	5	1
Malaise	8	7	Paresthesia	6	3
GASTROINTESTINAL			Somnolence	8	9
Anorexia	11	8	RESPIRATORY		
Diarrhea	12	18	Dyspnea	5	3
Dyspepsia	5	4	SKIN		
GI Pain	20	19	Rash	17	15
Nausea	46	18	SPECIAL SENSES		
Vomiting	6	3	Taste Perversion	5	8

Clinical adverse events which occurred in less than 5% of all patients treated with zidovudine are listed below. Since many of these adverse events were seen in placebo-treated patients as well as zidovudine recipients, they may not be attributable to the drug.

Body as a whole: body odor, chills, edema of the lip, flu syndrome, hyperalgesia, back pain, chest pain, lymphadenopathy.

Cardiovascular: vasodilation.

Gastrointestinal: constipation, dysphagia, edema of the tongue, eructation, flatulence, bleeding gums, rectal hemorrhage, mouth ulcer.

Musculoskeletal: arthralgia, muscle spasm, tremor, twitch.

Nervous: anxiety, confusion, depression, emotional liability, nervousness, syncope, loss of mental acuity, vertigo.

Respiratory: cough, epistaxis, pharyngitis, rhinitis, sinusitis, hoarseness.

Skin: acne, pruritus, urticaria.

Special senses: amblyopia, hearing loss, photophobia.

Urogenital: dysuria, polyuria, urinary frequency, urinary hesitancy.

DOSAGE AND ADMINISTRATION: The recommended starting dose of Retrovir (zidovudine) is 200 mg (two 100 mg capsules) administered orally every four hours around the clock.

Hematologic toxicities appear to be related to pretreatment bone marrow reserve and to dose and duration of therapy. Careful monitoring of hematologic indices every two weeks is recommended to detect serious anemia or granulocytopenia. In patients with hematologic toxicity, reduction in hemoglobin may occur as early as 2 to 4 weeks, and granulocytopenia usually occurs after 6 to 8 weeks.

Dose Adjustment: Significant anemia (hemoglobin of <7.5 g/dL or reduction of >25% of baseline) and/or significant granulocytopenia (granulocyte count of $<750/mm^3$ or reduction of >50% from baseline) may require a dose interruption until some evidence of marrow recovery is observed. For less severe anemia or granulocytopenia, a reduction in daily dose may be adequate. In patients who develop significant anemia, dose modification does not necessarily eliminate the need for transfusion. If marrow recovery occurs following dose modification, gradual increases in dose may be appropriate depending on hematologic indices and patient tolerance.

Wellcome
Burroughs Wellcome Co.
Research Triangle Park
North Carolina 27709

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HEMOSTASIS AND THROMBOSIS,
VOLUME 8

Edited by

Barry S. Coller, M.D.

Progress in Hemostasis and Thrombosis, Volume 8, carries on the tradition established for the series under Dr. Theodore Spaet's editorship, of publishing definitive reviews on major topics of current interest in the broad fields of hemostasis and thrombosis.

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Progress in Hemostasis and Thrombosis, Volume 8, presents hematologists, cardiologists, basic scientists, and other interested professionals, with in-depth reviews of important topics in the field.

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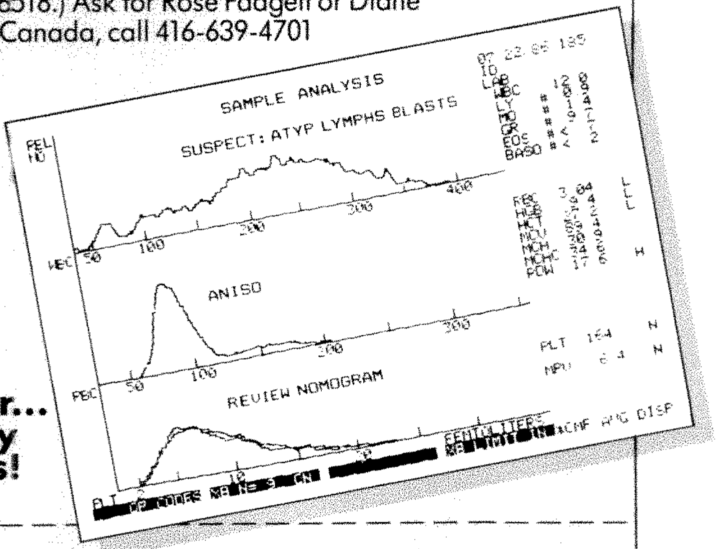

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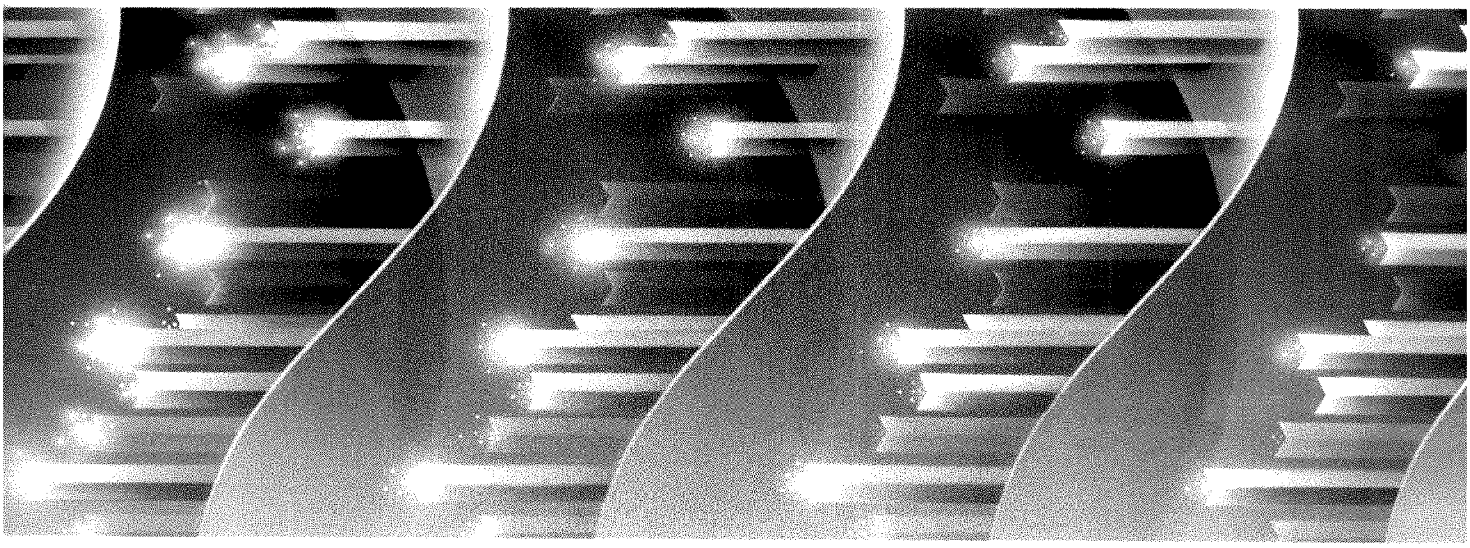

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REVIEW ARTICLE

Acute Myelogenous Leukemia: Recent Advances in Therapy

By Richard Champlin and Robert Peter Gale

WITHIN THE LAST DECADE there has been substantial progress in the treatment of acute myelogenous leukemia (AML). In this review we critically evaluate the role of remission induction and postremission chemotherapy, immunotherapy, biologic response modifiers, and bone marrow transplantation as well as innovative experimental treatments for AML.

AML results from malignant transformation of a hematopoietic progenitor cell followed by clonal proliferation and accumulation of the transformed cells. The pathogenesis of leukemia transformation is poorly defined but is likely to be a multistep process.[1,2] Fundamental to the acute leukemias is an apparent arrest in maturation early in the myeloid pathway, although some circulating granulocytes and other mature cells may be derived from the malignant clone.[2,3] In 10% to 20% of patients the leukemic cells have characteristics of both myeloid and lymphoid cells. These cases have been termed hybrid leukemias or mixed-lineage leukemias.[4-8] Hybrid leukemias may be biphenotypic, if a single cell displays both features, or bilineal, if different cells display myeloid and lymphoid characteristics. Bilineal leukemias can be synchronous or metachronous. Varied criteria have been used to document lymphoid and myeloid involvement. Lymphoid markers include morphology, surface antigens, terminal deoxynucleotide transferase, cytochemistry (PAS), and most recently, clonal rearrangement of Ig or T cell receptors genes. Myeloid markers include morphology (such as Auer rods and primary granules), electron microscopy, cytochemistry (Sudan black, myeloperoxidae, or nonspecific esterase), and most recently, cell surface antigens. Because of the considerable difficulty in the accurate diagnosis of hybrid leukemia we recently suggested possible criteria.[4] Hybrid leukemias appear to be more common in AML with a monocytic component and in patients with specific cytogenetic abnormalities, including the t(4;11) and t(9;22) translocations.[5,6] Biphenotypic leukemias may represent a malignancy of pluripotent stem cells or aberrent gene expression in neoplastic myeloid or lymphoid cells.

AML is a heterogeneous disease, differing considerably among patients in respect to cytogenetic abnormalities,[9-12] cellular phenotype,[13-16] and response to therapy. Acute leukemias do not usually perturb normal hematopoiesis or produce symptoms until a substantial burden of leukemia cells is present, generally 10^9 to 10^{12} cells.[17] At that point excess numbers of leukemia blasts are identified in the bone marrow, and leukemia cells enter the peripheral blood and infiltrate other tissues. The bone marrow is usually hypercellular but may be hypocellular in 5% to 10% of cases. Approximately one-quarter of patients develop AML following a myelodysplastic or preleukemic syndrome.[18] In these patients, normal hematopoiesis is suppressed by incompletely defined mechanisms.

In most instances of AML, normal progenitor cells persist in the bone marrow that can restore hematopoiesis after effective antileukemic therapy. Rarely, patients in what appears to be complete remission have evidence of clonal hematopoiesis[3,19] suggesting maturation of the malignant clone or restoration of a preleukemic state.

The goal of leukemia therapy is to eradicate the malignant cells. Treatment can be divided into several phases. The initial phase, termed remission induction, is designed to reduce the number of leukemia cells below the level of detection and restore normal hematopoiesis. By definition, no leukemia cells are detected in complete remission; however, substantial numbers of malignant cells probably persist in most patients[20] and lead to leukemia relapse if effective postremission therapy is not administered. A variety of postremission therapies have been proposed to eradicate residual leukemia. Unfortunately, resistance to treatment commonly develops, and most patients eventually relapse despite continued chemotherapy.

Bone marrow transplantation is increasingly used to treat patients with acute leukemia.[21-24] The major dose-limiting toxicity of many antileukemic drugs and radiation is bone marrow suppression. Doses of these agents can be substantially escalated if treatment is followed by bone marrow transplantation to restore hematopoiesis. Allogeneic bone

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marrow transplantation may also confer an immunologically mediated graft-*v*-leukemia effect.[25,26] Bone marrow transplantation has primarily been used as a form of consolidation treatment for patients in remission or to induce remission in patients with relapsed or resistant leukemia. Bone marrow transplantation may also be useful as initial therapy for selected patients with preleukemia or high-risk forms of acute leukemia unlikely to achieve remission with conventional therapy.[27,28]

Although the focus of this review is on therapy for AML, it is also important to use this data to analyze critically the conduct of clinical investigations in this field. The literature describing the treatment of leukemia is complex and difficult to interpret. Many contradictory studies have been reported. Most studies reporting the highest rates for remission induction or long-term survival involve selected patients and lack randomized or concurrent controls. Many exclude high-risk subgroups such as the elderly or patients with acute leukemia after a preleukemic syndrome.[29] In other studies such as those involving intensive postremission chemotherapy or bone marrow transplantation for patients in first remission, treatment results are distorted by selection in favor of patients in good overall condition and in remission for several months, with the exclusion of patients who relapse rapidly or have persistent infections or other medical complications. One frequent problem in the interpretation of therapeutic data in acute leukemia relates to the uncertainty in accepting the results of small, uncontrolled trials with inadequate follow-up. Preliminary results of studies suggesting a therapeutic advance are more likely to be prominently published than negative findings that may not be reported. Results published from single-institution studies generally exceed those reported by large cooperative groups utilizing similar therapies. Thus, caution is needed in the interpretation of uncontrolled studies, and results must be considered in relation to patient selection and prognostic factors.[30]

Results of identical treatment protocols may vary substantially between centers or over time. For example, the Cancer and Leukemia Group B utilized the same remission induction chemotherapy in two consecutive trials; the remission rates differed substantially although the therapy was identical.[31] Recently, patients entered into a large Medical Research Council trial in its second half had a significantly higher remission rate than individuals entered into the first half of the study, again despite identical treatment.[32] These results could not be accounted for on the basis of known prognostic factors and emphasize that variables other then chemotherapy can influence the outcome of drug trials in AML. This observation suggests strongly that therapeutic trials should be prospective and randomized; trials not fulfilling these criteria must be viewed cautiously. In this review we focus, when possible, on the results of prospective controlled trials.

REMISSION INDUCTION CHEMOTHERAPY

The cornerstone of remission induction chemotherapy is the combination of cytarabine[33,34] and an anthracycline.[35,36] Few randomized studies comparing drug doses or different anthracyclines have been reported. The best results utilized cytarabine, 100 to 200 $mg/m^2/d$, by constant or intermittent intravenous infusion for seven days in combination with daunorubicin, 30 to 70 $mg/m^2/d$, by intravenous bolus on the first or last three days.[31,37-44] Addition of 6-thioguanine or other agents has not proved beneficial.[45] Patients who fail to achieve complete remission with one or two courses of this induction chemotherapy have a poor prognosis; even if remission is ultimately achieved, it is typically brief.[44] This conclusion may not apply for less intensive regimens; five-day treatment schedules have also been effective in some studies but generally require multiple courses of treatment to achieve remission.[32,46,47] Approximately half of patients who fail to achieve remission have resistant residual leukemia, and the remaining half of patients with treatment failures die of infections, bleeding, or rarely, direct toxicity of the chemotherapy.[48,49]

A recent trial comparing 100 *v* 200 mg/m^2 of cytarabine given by continuous intravenous infusion showed no difference in remission rates.[50] Daunorubucin is preferable to doxorubicin in induction chemotherapy, since its use is associated with less mucosal and gastrointestinal toxicity.[36,39] The optimal dose of daunorubicin is uncertain. The drug is generally well tolerated in doses up to 60 to 75 $mg/m^2/d$ for three days in young patients, but the dose should probably be reduced in patients over 60 to 70 years of age. Except for patients with preexisting cardiac dysfunction, cardiotoxicity is unusual with cumulative daunorubicin doses <1 g/m^2.[51] Combinations of cytarabine with either amsacrine[42,52] or mitoxantrone[53] appear as effective as with daunorubicin. Mitoxantrone appears to produce less mucositis than anthracyclines and is better tolerated by older patients. Several investigational anthracyclines, including idarubicin,[54] aclarubicin,[55] and epidoxorubicin[56] are currently under investigation. Some may have advantages with regard to cardiac toxicity, but most data suggest cross-resistance with daunorubicin. In one recent radomized trial, the combination of cytarabine and idarubicin produced a higher remission rate than cytarabine and daunorubicin.[57]

Some investigators have evaluated more intensive regimens such as extending cytarabine administration to ten days.[49] Although selected patients may benefit, this approach results in increased toxicity and a greater rate of early death without a clear improvement in the remission rate or duration. As a consequence, its routine use cannot be recomended.

High-dose cytarabine alone, with L-asparaginase, an anthracycline, amsacrine, or mitoxantrone has recently been evaluated as remission induction therapy for patients with AML;[58-62] it is not certain whether these additional drugs increase efficacy. In one recent study, the remission rate with high-dose cytarabine alone exceeded 90%.[62] Results are variable; although some uncontrolled studies report relatively high remission rates, there is no convincing evidence that survival is improved compared with that achieved with standard-dose cytarabine with daunorubicin. In addition, because of the cumulative toxicity of high-dose cytarabine, its inclusion for inducttion chemotherapy may limit its use during postremission treatment.

Several patient groups pose particular problems in remission induction treatment. Patients greater than 60 years of age generally have lower remission rates.[31,32,39,43,63,64] This appears to be primarily due to a greater risk of early death from infection or toxicity, but disease-related factors may also be important. Elderly patients have a higher incidence of preleukemic syndromes and are more likely to have cytogenetic abnormalities associated with a poor prognosis. In two randomized studies, elderly patients receiving attenuated doses of cytarabine and daunorubicin had better results than with full-dose regimens.[39,65]

Patients in whom AML develops after a preleukemic or myeloproliferative syndrome[66-68] or after exposure to cytotoxic chemotherapy and/or radiation given for other malignancies (therapy-linked AML)[69] have only a 20% to 40% likelihood of achieving remission with standard induction chemotherapy. Other patients, usually elderly, present with slowly progessive or "smoldering" acute leukemia; they also have a lower remission rate with intensive therapy.[70] Low-dose cytarabine, 10 to 20 mg/m^2/d, has been used to treat elderly patients and patients with poor-risk categories of acute leukemia;[71-73] durable remissions are unusual, and most patients require at least 3 weeks of treatment, which typically results in bone marrow aplasia. There are no convincing data that low-dose cytarabine is as effective as conventional-dose induction chemotherapy for these high-risk patients, and its use is not recommended if the objective of therapy is to achieve a remission. High-dose cytarabine has also been evaluated in patients with therapy-linked AML[74] or other poor prognostic subgroups. Although preliminary encouraging results wre reported, there is no clear evidence that this approach is superior to other induction regimens.

The management of patients who develop acute leukemia during pregnancy poses special problems. The potential teratogenicity of chemotherapy is of concern during the first trimester; the incidence of fetal malformations does not appear to be increased with chemotherapy given after this time.[75-77] Bleeding is an additional problem, particularly during labor, since chemotherapy produces thrombocytopenia in the fetus as well as the mother. It is often appropriate to delay chemotherapy treatment in patients presenting in the third trimester until after delivery. Leukopheresis may be useful as a temporary measure.[78]

POSTREMISSION CHEMOTHERAPY

Further cytoreduction is probably required in patients who achieve remission to eradicate residual leukemia cells and prevent relapse. Several terms have been utilized to describe forms of postremission chemotherapy. Unfortunately, these terms are poorly defined, and their use has not been uniform. *Consolidation* or *early intensification* refers to one or more courses of therapy administered soon after achieving remission that produces severe myelosuppression. *Late intensification* involves one or more courses of similarly intensive chemotherapy but given after a delay of 6 to 12 months. *Maintenance* chemotherapy is generally conceived of as frequent courses resulting in less severe myelosuppression given over several months or years. The efficacy of these therapies in prolonging remission duration and in preventing relapse is controversial.[79-82]

The median duration of remission and proportion of long-term survivors after modern remission induction chemotherapy alone is largely unknown, since almost all contemporary studies have utilized some form of postremission therapy. In one study patients induced into remission with cytarabine and daunorubicin received no further therapy. The median remission duration in 19 patients was 8 months; 12% remain disease free beyond 5 years.[83] In a subsequent trial, a single postremission cycle reportedly increased the median remission duration to 30 months; this study was not randomized, and it is difficult to draw meaningful conclusions. Preliminary results were recently reported from an Eastern Cooperative Oncology Group study that randomized patients entering remission to no further therapy, standard maintenance, or high-dose intensive consolidation.[84] The no-therapy arm was terminated because of rapid leukemia relapses and a median remission duration of only 4 months; this was significantly inferior to the maintenance therapy group. The consolidation chemotherapy group was not analyzed. These studies demonstrate that some form of postremission therapy is indicated to prolong remission duration.

Studies reporting the longest remission durations have utilized two or more courses of consolidation/intensification chemotherapy;[32,44,85-89] the median remission duration has generally ranged from 1 to 2 years with 5-year disease-free survival rates of 10% to 25% in adults and up to 45% in children.[41,90] Most studies involve two to four courses of intensive consolidation therapy using similar agents for induction. Several recent studies using high-dose cytarabine alone or with an anthracycline as consolidation have produced encouraging results with approximately 40% 3- to 5-year leukemia-free survival rates.[59,91-93] These preliminary results require confirmation. Several randomized controlled studies of consolidation chemotherapy have been reported.[32,94-96] These studies show a trend toward improved disease-free survival, but this has not been statistically significant in most instances.

Maintenance chemotherapy in AML has generally utilized repeated short courses of intravenous or subcutaneous cytarabine with thioguanine or daunorubicin given monthly for 2 to 3 years. Numerous controlled and uncontrolled trials with few exceptions have failed to demonstrate a substantial benefit in patients receiving maintenance therapy compared with those receiving induction and consolidation alone.[44,85,86,97-101] A recent multicenter German study reported superior results with maintenance therapy, but this was probably due to unexpectedly poor results among controls.[102] These data are similar to results for treatment of most other human cancers where maintenance chemotherapy generally does not prolong survival. Maintenance chemotherapy should not be routinely utilized in the treatment of AML in patients receiving intensive induction and consolidation chemotherapy.

Late intensification chemotherapy has also been reported to improve the remission duration in uncontrolled studies.[103,104] Children receiving protracted regimens involving repeated cycles of intensive chemotherapy have had favorable results in some[41,90] but not all studies. Adults generally do not tolerate these regimens as well and have not appeared to benefit beyond results achieved with initial consolidation alone. Results of recent randomized trials did, however, indicate superior remission duration in adults receiving late intensification chemotherapy 1 year after achieving complete remission, although this did not reach statistical significance.[32] There is considerable controversy regarding the total length of treatment and the value of late intensification for patients with acute myelogenous leukemia.

DIFFERENTIATING AGENTS

An innovative potential approach to the treatment of leukemia is to induce maturation of leukemic blasts to terminal, nonproliferative cells.[105] Several agents can induce leukemia cells to differentiate in vitro, including phorbol esters, dimethylsulfoxide, and retinoids.[106] Retinoic acid has been studied clinically in patients with smouldering leukemia or preleukemia, but with rare exceptions, it has not been beneficial in these settings or in typical AML.[107,108] Low doses of many chemotherapy drugs are weak inducers of cellular differentiation. Low-dose cytarabine has been proposed to act by this mechanism,[71] but in most patients, it appears to act through cytotoxicity to produce a hypocellular bone marrow after 2 to 3 weeks of treatment.[72,73]

IMMUNOTHERAPY AND BIOLOGIC RESPONSE MODIFIERS

There have been extensive attempts to utilize immunologic agents to treat AML. Initial studies utilized adjuvants and nonspecific agents such as bacillus Calmette-Guerin,[103,109,110] *Cornybacterium parvum,*[35] or other agents alone or in combination with irradiated or chemically modified autologous or allogeneic leukemia cells in an attempt to enhance antileukemic immunity. This form of immunotherapy alone or with maintenance chemotherapy has generally failed to prolong the remission duration.[111,112]

Human leukocyte interferon and recombinant α-interferon have also been evaluated but have not shown activity.[113] Intravenous monoclonal antibodies reactive with AML cells have also been studied; transient reduction in circulating blasts may occur, but there is little effect on the bone marrow, and complete remissions have not been reported.[114] Preliminary trials with tumor necrosis factor and with combinations of interferons are in progress.

TREATMENT OF THE CENTRAL NERVOUS SYSTEM

CNS involvement in AML is generally due to infiltration of leukemia cells into the meninges. CNS leukemia is uncommon at diagnosis. Ultimately, 5% to 10% of patients will have meningeal involvement, usually in the setting of bone marrow relapse.[32,115-117] Because of this, prophylactic therapy to the CNS is generally not indicated. CNS relapse occurs most frequently in patients with myelomonocytic or monocytic subtypes; some centers recommend prophylactic local treatment in children with these subtypes.[117] Treatment of documented CNS relapse usually uses intrathecal cytarabine and/or methotrexate alone or combined with cranial radiation. Although this approach can provide local control, leukemia is likely to recur; more typically, systemic relapses intervene.

PROGNOSTIC FACTORS IN AML

Numerous studies have attempted to define clinical factors predictive of either remission induction or duration.[67,118-121] Potential factors involve patient- and disease-related variables. The primary patient-related factor is age.[29,31,32,62-64] Elderly patients generally have lower remission rates, but in those achieving remission, its duration is not substantially different than that in young adults.[39,44,119] Disease-related factors include histologic subtype,[121,122] presence of a preleukemic syndrome,[66,67] and evidence of extramedullary disease. Cytogenetic abnormalities may be fundamental to the pathogenesis of acute leukemia and also provide independent prognostic information.[9-12] Characteristic chromosome abnormalities correlate with histologic subtypes as summarized in Table 1. Patients with t(8;21), t(15;17) and inversions or translocations of chromosome 16 have a relatively good prognosis, whereas those with -5/5q-, -7/7q-, t(4;11), t(6;9), t(9;22) tend to have a poor outcome.

The French-American-British (FAB) histologic subtype classification is of limited prognostic significance.[121,122] The promyelocytic (M_3) subtype is associated with a coagulopathy due to disseminated intravascular coagulation and often excessive fibrinolysis that may lead to bleeding complications during induction chemotherapy.[123-127] Prophylactic heparin is widely used to suppress intravascular coagulation; although no prospective randomized trials have been performed, uncontrolled trials suggest that heparin reduces bleeding complications.[123,124,126] Release of proteases from leukemic promyelocytes may inactivate fibrinolytic inhibitors such as α-2-plasmin inhibitor and lead to excessive fibrinolysis. Treatment with the combination of ϵ-aminocaproic acid and heparin may be effective to prevent bleeding in patients with acute promyelocytic leukemia and reduce the levels of α-2-plasmin inhibitor.[127] Patients with promyelocytic leukemia who achieve remission may have a longer remission duration and a longer survival time than patients in other subgroups, but this is controversial.[123,124] Patients with Auer rods (M_2 or M_3 subtype) tend to have a better prognosis than those with myelomonocytic, monocytic, or erythroid subtypes.[67,99,121,122] Acute megakaryocytic leukemia, recently defined as the

Table 1. Correlation of Chromosome Abnormalities With Histologic Subtypes

Chromosome Abnormality	Histologic Subtype	Relative Prognosis
t(8;21)	M_2	Good
t(15;17)	M_3	Good*
t(9;11) or 11q–	M_4, M_5	Poor
inv or del (16)	M_4, M_5	Good
t(6;9)	M_2, M_4	—
+8	M_1, M_2, M_4, M_5, M_6	—
–5,5q–, –7,7q–	Treatment induced or evolving from preleukemic syndrome	Poor
t(9;22)-Ph^1	M_1, M_2, M_4, M_5, M_6	Poor
t(4;11)	Hybrid; M_5	Poor
Aneuploid or pleomorphic	M_1, M_2, M_4, M_5, M_6	Poor

*Associated with disseminated intravascular coagulation during remission induction; may have longer duration of remission.

FAB M_7 variant,[128] may have a relatively poor prognosis.[129] Parameters related to the mass of leukemia cells such as WBC and lactic dehydrogenase, the mitotic or labeling index, abnormalities in myeloid colony formation, and response to chemotherapy in clonogenic assays in vitro have been predictive in some but not all reports and are of limited clinical utility.[130-134]

The clinical signficance of the hybrid leukemias with both myeloid and lymphoid characteristics is unknown.[4,8] Present data suggest that these individuals, particularly those with the t(4;11) or t(9;22) translocation, are less responsive to chemotherapy and have a lower probability of long-term disease-free survival. Prospective trials are needed to confirm this impression. In the interim, most centers treat these individuals with intensive chemotherapy, and CNS prophylaxis is sometimes given. It has also been recommended that these individuals might be candidates for bone marrow transplantation. Trials comparing chemotherapy or transplantation in this setting have not been reported.

Several investigators have used combinations of these factors to formulate a prognostic index with the goal of identifying good- and poor-risk prognostic groups and to define patients who should receive experimental therapies or bone marrow transplantation.[119] Although these indexes can distinguish some prognostic groups, it is currently impossible to reliably identify patients who have more than a 50% chance of prolonged survival with chemotherapy. These indexes should probably not be used to assign treatment except in the context of controlled trials.

TREATMENT OF RELAPSED AML

Despite optimal initial therapy, a substantial proportion of patients ultimately relapse. The chance of achieving a second remission depends on the nature of prior treatment, the length of the first remission, and the initial response to therapy. Twenty-five percent to 50% of patients will achieve a second remission after treatment with cytarabine and daunorubicin, amsacrine, or mitoxantrone.[32,44] Extensively treated patients are less likely to respond. The median duration of second remissions is usually less than 6 months, and there is no evidence that additional chemotherapy is effective in prolonging the duration of remission.

High-dose cytarabine alone[135-137] or in combination with L-asparaginase,[58] an anthracycline,[138] amsacrine[139] or mitoxantrone[140] may increase the remission reinduction rate to 50% to 70%. The optimal dose and schedule of high-dose cytarabine are uncertain.[141] Doses of 3 g/m^2 over one to three hours every 12 hours for four to six days have been commonly utilized, but comparable results have been reported in some studies using lower doses.[62,142] Higher doses result in greater toxicity. The risk of irreversible cerebellar dysfunction increases with the dose and duration of therapy.[143]

Other single agents have limited activity in recurrent AML. Amsacrine,[52,144] 5-azacytidine,[145] mitoxantrone,[53,146] homoharringtonine,[147] diaziquone,[148] cyclocytidine,[149] etoposide,[150] experimental anthracycline analogues,[54-56] or combinations of those agents[151-153] produce transient remissions in less than 30% of patients. Two promising regimens appear to be mitoxantrone with cytarabine[140] or with etoposide.[153]

BONE MARROW TRANSPLANTATION

High-dose chemotherapy and radiation combined with bone marrow transplantation from an HLA-identical sibling is an effective treatment for AML and is the preferred therapy in selected patients.[21-24] Results are primarily related to the disease status at the time of transplantation; these data are reviewed in Table 2. Initial transplant studies involved patients with advanced resistant disease who failed to respond to standard and investigational chemotherapy. Ten percent to 20% long-term disease-free survival rates were achieved even in this unfavorable setting. Major causes of treatment failure were leukemia relapse and transplant-related complications such as toxicity, graft-*v*-host disease, interstitial pneumonitis, and opportunistic infections. Improved results have been reported in patients who received transplants earlier in the course of their disease. Approximately 30% achieve prolonged survival if transplants are performed at the time of initial relapse or in a second remission; there appears to be no advantage in administering further chemotherapy or attempting to achieve a second remission before bone marrow transplantation.[154]

The best results of bone marrow transplantation in AML have been achieved in patients who received transplants while in first remission;[155-158] actuarial relapse rates vary from 0% to 40%, and 40% to 70% of patients achieve prolonged, disease-free survival. Outcome is related to patient age in most studies,[158-161] with 50% to 70% survival rates in patients under the age of 30 and 30% to 45% survival rates for patients 30 to 50 years of age. Patients over 50 years of age are not routinely considered for bone marrow transplantation.[161]

Most centers utilize a pretransplant preparative regimen of high-dose cyclophosphamide and total body radiation. Some centers have evaluated alternative preparative treatments with the goal of reducing the risk of relapse. The regimen of busulfan and cyclophosphamide[162] or combining high-dose cytarabine[92,163] or etoposide[164] with total body radiation may provide more effective antileukemic therapy, but the overall survival is similar to other regimens. Controlled trials comparing these regimens have not been reported.

It is important to understand the mechanism of relapse after bone marrow transplantation. The vast majority of patients who relapse have recurrence in recipient cells, which indicates an inadequate antileukemic effect. In rare patients, particularly those relapsing several years posttransplant, leukemia recurrence has been reported in donor cells.[165]

Table 2. Comparison of Disease-Free Survival Reported for Bone Marrow Transplantation and Chemotherapy in AML

	BMT	Chemotherapy
Refractory relapse	10	0
Initial relapse	20-30	<5
Second remission	20-50	<5
First remission		
Age <30 yr	50-70	20-50
Age 30-50 yr	30-45	10-45

Values approximate actuarial survival ≥3 years.

Abbreviation: BMT, bone marrow transplantation.

These data suggest reinduction of leukemia; alternative explanations are also possible, including transfection of oncogenic DNA or, in the case of sex-mismatched transplants, exchange of X and Y chromosomes between a residual host leukemia cell and a donor cell.

There is considerable controversy whether patients with AML in first remission who have an HLA-identical sibling should receive a bone marrow transplant at that time or whether the transplant should be delayed until relapse or a second remission. In patients <30 years of age, results of bone marrow transplantation are equivalent or superior to those achieved with chemotherapy, and its use is reasonable. Representative results for adults are illustrated in Fig 1. Three controlled studies have been reported in adults[99,166,167]; in each study there was a significantly lower rate of leukemia relapse with bone marrow transplantation. The overall survival was higher with transplantation in each study, but the difference was significant in only one[99] because of a higher rate of treatment-related complications with bone marrow transplantation such as graft-*v*-host disease and interstitial pneumonia. When one also considers that 20% to 30% of patients who relapse after postremission chemotherapy can be salvaged by bone marrow transplantation, it remains uncertain whether early bone marrow transplantation is warranted in adults in first remission (Fig 2). This question requires examination in a prospective controlled study. Based upon current data, the routine use of bone marrow transplantation cannot be recommended for patients in first remission who are older than 30. Some investigators have suggested the use of prognostic factors to identify individuals most likely to benefit from transplant *v* chemotherapy. For example, patients with the monocytic subtype or with a high initial leukocyte count have poor results with chemotherapy. Unfortunately, these biologic factors also predict an unfavorable outcome with bone marrow transplantation.[168]

Advances in bone marrow transplantation may alter these conclusions in the future. The use of cytomegalovirus-seronegative blood donors[169] and prophylactic intravenous immunoglobulin[170] may reduce the incidence of interstitial pneumonitis. Removal of T lymphocytes from the donor bone marrow can prevent graft-*v*-host disease; unfortunately the rate of graft rejection and the risk of leukemia relapse are increased.[171,172] These problems may potentially be overcome by more effective preparative regimens, but it remains to be determined whether T cell depletion will improve survival.[173]

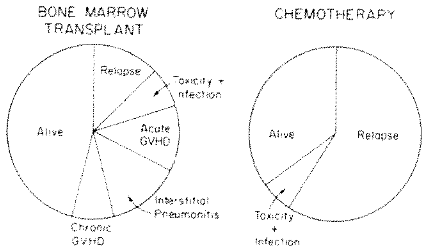

Fig 1. Representative results of bone marrow transplantation from an HLA-identical donor (data from the International Bone Marrow Transplant Registry) *v* postremission chemotherapy for adult patients with AML in first remission.

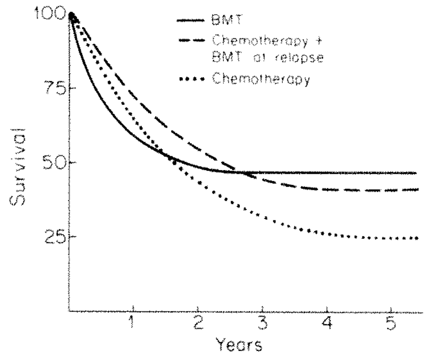

Fig 2. Representative results of bone marrow transplantation and post remission chemotherapy for adult patients with AML. The dashed line indicates projected survival for patients initially receiving postremission chemotherapy with bone marrow transplantation as salvage therapy at the time of relapse. Reprinted with permission.[21]

Other methods to reduce graft-*v*-host disease are also promising; posttransplant immunosuppressive treatment with the combination of cyclosporine and methotrexate is more effective than either drug alone, and extended disease-free survival has been reported in up to 70% of patients treated with this regimen.[174]

Most patients do not have an HLA-identical sibling. As a consequence there has been considerable interest in the use of alternative donors or sources of hematopoietic stem cells for transplantation. Bone marrow transplants from related, partially HLA-matched donors have been reported.[175-177] Both the risk of graft rejection and graft-*v*-host disease increase progressively with greater genetic disparity between the donor and recipient.[176-178] Preliminary data suggest that the survival of patients receiving transplants from related donors phenotypically HLA identical or mismatched for only one HLA-A, -B, or -D locus antigen is similar to that with HLA genotypically identical grafts.[176,177] Results in recipients of two or three locus HLA-mismatched transplants are poorer, particularly in adults. Some centers have attempted T cell–depleted transplants from HLA-haploidentical donors with the goal of preventing graft-*v*-host disease. Preliminary data indicate a high rate of graft rejection and graft-*v*-host disease with few survivors.[172,179]

Bone marrow transplants from unrelated, HLA-identical donors have also been attempted.[177,180-182] The considerable polymorphism of HLA loci makes this approach difficult. Preliminary data indicate that successful transplants are possible, that the risks of graft rejection and graft-*v*-host disease are considerable, and that survival is <20%. A very large registry of potential donors is required to identify completely HLA-matched donors.[183] Registries of this type exist in North America and Europe. The North American registries are currently being organized into a centralized network. We recently projected the potential impact of this approach in AML if this strategy were successful.[184]

AUTOLOGOUS BONE MARROW TRANSPLANTATION

Autologous bone marrow transplantation using bone marrow cells or, more rarely, peripheral blood cells collected and cryopreserved while the patient is in remission has also been considered in patients with AML. At a later time, the patient receives high-dose chemotherapy with or without total body irradiation followed by reinfusion of the stored bone marrow cells. The major limitation of this approach is that the "remission" bone marrow is likely to be contaminated by occult leukemia cells. In addition, any graft-*v*-leukemia associated with allogeneic bone marrow transplantation would probably not occur with autologous transplantation. There has been a high rate of leukemia relapse after autologous bone marrow transplantation in patients with advanced AML.[185] Several approaches have been proposed to eradicate residual leukemia cells from autologous bone marrow, including treatment with monoclonal antibodies reactive with leukemia cells or pharmacological agents. Unfortunately, most antibodies reactive with myeloid leukemia cells also cross-react with normal hematopoietic progenitors.[15,16] The considerable heterogeneity of leukemia cells makes it uncertain whether one antibody will react with all clonogenic leukemia cells. Recent data suggest that the phenotype of leukemia stem cells may differ considerably from their progeny.[14] Nonetheless, a pilot study reported hematologic recovery and remissions of up to 21+ months in AML patients who received transplants in relapse or in second remission with autologous bone marrow treated with anti-AML monoclonal antibodies.[186] Two pharmacological agents related to cyclophosphamide, 4-hydroperoxycyclophosphamide (4HC)[187] and mafosfamide (ASTA-Z),[188] have been used to eliminate leukemia cells from autologous bone marrow. In one recent report[187] 11 of 25 (42%) patients receiving autologous transplants of 4-HC–treated bone marrow while in second or third remission survived more than 1 year free of relapse; these encouraging results would not be expected with alternative therapies. A number of patients with AML have recently received autologous bone marrow transplants using untreated or mafosphamide-treated bone marrow while in first remission[188,189]; unfortunately these studies were uncontrolled and involved highly selected patients so that critical evaluation of the results is not possible. An innovative method to remove leukemia cells from autologous bone marrow involves maintaining the bone marrow in long-term culture; this may allow a growth advantage for normal cells.[190] Further studies are required to determine whether this approach is effective.

Autologous bone marrow transplantation remains an experimental approach. Further carefully conducted controlled trials are necessary to assess the efficacy of this technique and its relative role compared with alternative therapies. It is possible to envision this approach as a form of intensive postremission therapy in which one elects to give extremely high dose treatment to the vast majority, but not all, of the bone marrow *v* less intensive therapy to the entire bone marrow with conventional chemotherapy.

SUMMARY

Results of treatment of AML have improved over the last decade. With modern remission induction chemotherapy, most patients achieve complete remission. The median remission duration is now 1 to 2 years, and a substantial fraction of patients achieve long-term disease-free survival. Bone marrow transplantation provides an improved antileukemic effect compared with chemotherapy alone, and it is the treatment of choice for selected groups of patients. The major limitation is the risk of transplant-related mortality. Most patients are not currently eligible for bone marrow transplants because of advanced age or lack of a histocompatible donor. Autologous marrow transplantation may be effective in selected settings, but this remains investigational at present. Substantial improvement in results of treatment of AML requires the development of new and effective chemotherapeutic agents that are non–cross-resistant with available drugs. Results of bone marrow transplantation may substantially improve if innovative measures to prevent major complications are successful.

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Vasoactive Amines Directly Modify Endothelial Cells to Affect Polymorphonuclear Leukocyte Diapedesis In Vitro

By John Doukas, David Shepro, and Herbert B. Hechtman

Bovine aortic endothelial cells were cultured on the basement membrane surface of amnionic membrane and used as a substrate for polymorphonuclear leukocyte (PMN) diapedesis in vitro. Norepinephrine (NE), serotonin (5HT), or phalloidin treatment of the endothelial cells (ECs) reduces, whereas histamine or cytochalasin B increases, the number of PMNs migrating across the ECs and amnionic membrane. In contrast, amine treatment of PMNs or acellular amnionic membrane does not alter PMN diapedesis or chemotaxis. The NE and histamine effects are blocked by appropriate receptor antagonists, but the 5HT effect is not. All the agents' effects are also reversible. Qualitatively similar effects on EC permeability to Evan's blue-labeled albumin occur with all agents; however, PMN adhesion to ECs is not affected. Previously, we reported that NE and 5HT increase stress fiber numbers and decrease EC permeability to macromolecules in vitro, whereas histamine has the opposite effects, and that NE and 5HT eliminate the erythrocyte extravasation associated with thrombocytopenia in vivo. In this study, we propose that these vasoactive amines also alter PMN diapedesis in vitro through a direct effect on the EC, in part due to alterations in the EC cytoskeleton.

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THE PROCESS of polymorphonuclear leukocyte (PMN) diapedesis, in which PMNs breach the endothelial cell (EC) barrier of the microvasculature and enter surrounding tissues, is of prime importance in the development of an inflammatory reaction.[1] PMN diapedesis has been examined principally with regard to the earlier event of adherence to the endothelium,[2,3] or with regard to PMN adhesion to foreign surfaces[4] and chemokinesis.[3,5] In the present study, however, we examine an active role for ECs in PMN diapedesis which, if demonstrated, would suggest that local metabolic regulation of ECs may help to maintain the microvascular barrier to PMNs.

We previously reported that vasoactive amines such as norepinephrine (NE), serotonin (5HT), and histamine modulate various EC functions such as permeability to macromolecules,[6] the erythrocyte extravasation associated with severe thrombocytopenia,[7] and surface area and stress fiber numbers.[8] Stress fibers are microfilament bundles composed of actin, myosin, and other contractile proteins,[9] which in ECs are believed to assist in maintaining normal barrier function to fluid and macromolecular permeability.[10-14] An underlying hypothesis in our work is that exogenous metabolites such as NE and 5HT act as local regulators to maintain the normal barrier function of EC.

PMN diapedesis has been studied using pulmonary artery intimal explants[15] as well as ECs cultured on glass[16] and polycarbonate filters.[17] These systems, however, have inherent limitations; for example, intimal explants retain a portion of the vessel wall, which can alter expected permeability changes[18]; cultures on glass coverslips do not possess a subendothelial connective tissue layer to be crossed by PMNs as occurs in vivo, and monolayers cultured on filters may show areas of attenuated cytoplasm overlying filter pores,[17] an aberrant growth pattern not seen in ECs in vivo or cultured on substrates without pores. A more suitable substrate for use in PMN diapedesis studies is the acellular amnionic membrane, which provides normal EC monolayer growth over a continuous, native basement membrane and collagenous interstitial stroma.[19] In this report, using ECs cultured on amnionic membrane mounted in modified chemotaxis chambers, we observed that the amines NE and 5HT significantly and reversibly decreased PMN diapedesis in vitro whereas histamine increased diapedesis through their direct effects on the EC.

MATERIALS AND METHODS

Tissue culture. Calf aortas were collected shortly after slaughter and transported on ice. After excess tissue was trimmed, the lumens were rinsed with phosphate-buffered saline (PBS) and exposed, and the intima was removed by gentle pressure with a scalpel. Individual cells were dispersed by treatment with 0.1% collagenase in Ca^{2+}, Mg^{2+}-free PBS at 37°C for 12 minutes. After centrifugation at 1,200 g for 4 minutes, the cell pellet was suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL), and plated at 1 to 2 aorta/25-cm^2 flask. After 12 hours, debris was removed by rinsing the pellet with fresh medium. Cultures were refed every 3 days, and confluent monolayers were obtained in 5 to 7 days. ECs were confirmed by morphology, failure to overgrow as a monolayer, and positive staining for low-density lipoprotein (LDL) receptors using a fluorescent-labeled acetylated LDL (DiI-Ac-LDL).[20]

Amnionic membrane preparation. A modification of the method of Liotta and colleagues[21] was used to prepare acellular amnionic membrane. Human placentas were obtained shortly after delivery and transported on ice. The amnion reflecta of the sac material was separated from the chorion, washed free of blood, and placed in two changes of 1 mmol/L of *N*-ethyl maleimide at 4°C over 60 minutes, with gentle agitation. The tissue was rinsed in 4°C distilled water, stretched loosely over plastic embroidery hoops, and the excess gelatinous stroma was removed by gentle rubbing. The hoops were submersed in 25°C 2% NH$_4$OH for 30 minutes, with gentle agitation, after which the epithelium was removed with a rubber policeman. The amnionic membrane was then rinsed in distilled water and stored in 4°C distilled water for up to 12 hours prior to use.

PMN preparation. Heparinized human blood was obtained from healthy, drug-free donors and immediately used for isolating

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PMNs, using discontinuous density gradients of Ficoll-Hypaque.[22] Contaminating erythrocytes were then removed by centrifugation (200 g for 3 minutes) through 3% Dextran T500 in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS), and the PMNs were rinsed free of gradient fluid with HBSS (200 g for 5 min), and resuspended in buffer (DMEM containing 0.45% BSA, pH 7.4) at a final concentration of 6.25×10^6 cells/mL. This procedure yielded >97% PMNs (Wright's-Giemsa staining), with >98% viability as assessed by trypan blue exclusion.

Permeability chamber assembly. Amnionic membranes were arranged in modified chemotaxis chambers constructed in our laboratory from plastic test tube caps (Fig 1). Hoops of membrane, basement membrane side up, were placed on top of the open ends of lower caps, which had circular inner ridges extending 2 mm upward from the cap floors. Upper caps, which had outwardly flaring bases 1.5 mm thick, were pressed into the lower caps base first. In this way, upper and lower wells were formed (250-μL capacity each), separated by 50-mm^2 circles of amnionic membrane held firmly in place between the bases of the upper caps and the ridges of the lower caps. The caps themselves fit tightly into each other, holding the assemblies firmly in place. The tops of the upper caps had been previously cut open to allow addition of cells and medium onto the basement membrane (formerly the epithelial surface) of the amnionic membrane; the lower wells were closed except for small holes previously drilled in their sides to allow emptying and refilling. After assembly, excess membrane was trimmed, the upper wells were filled with complete tissue culture medium (DMEM with FBS and antibiotics) and, if no leakage into the lower wells occurred due to a membrane tear, the lower wells were likewise filled by a syringe inserted through the side holes. The chambers were placed in 24-well tissue culture plates, the plates were filled with medium so as to cover the side holes of the chambers, and the plates were sterilized by exposure to a ^{60}Co source for 3 to 5 minutes. The chambers were then stored at 4°C for 1 to 2 weeks until needed.

Bovine aortic endothelial cell (BAEC) monolayers were trypsinized and seeded onto the basement membrane surface of amnionic membrane at a concentration of 1×10^5 cells/chamber, at which time fresh complete medium was also added. This seeding density produced visual confluency within 12 hours; however, for maximum density and integrity, the monolayers were cultured for an additional 48 hours prior to use, with a medium change at 24 hours. For assessment of confluency, representative monolayers were stained with saturated auramine *O* (aqueous) for 1 minute and viewed by epifluorescence with a mercury light source. This staining procedure clearly delineates the cells in a confluent monolayer and any gaps that may be present; it was developed because the collagen fibrils of the amnionic stroma coupled with the plastic of the permeability chambers prevented effective transillumination.

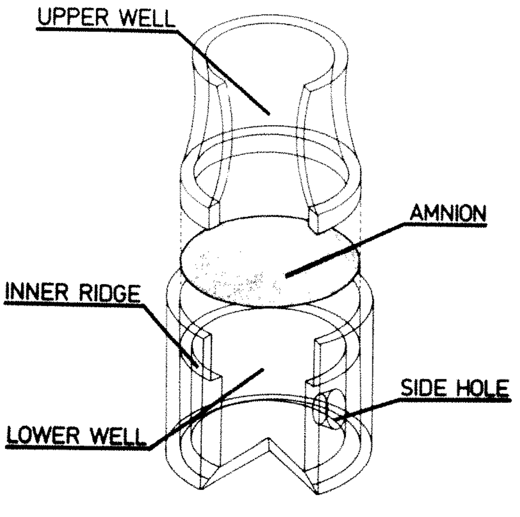

Fig 1. Permeability chamber diagram.

Permeability chambers constructed in this manner routinely yielded comparable levels of PMN diapedesis. In addition, with Evan's blue-labeled albumin used as a permeability tracer, the monolayers routinely demonstrated comparable barrier function to this macromolecule. The final passage number of the monolayers (passage 1, 2, or 3) did not affect these results. Transmission electron micrographs (not shown) reveal a continuous monolayer, an underlying basement membrane, and an interstitial stroma of collagen fibrils devoid of rents or tears, as has been previously described by Madri and Williams.[19] In experiments in which membrane tears were purposely created, a greatly increased passage of PMNs into the lower well (~50% of all PMNs applied *v* the average of 8% for normal control conditions) or of Evan's blue-labeled albumin (EBA) occurred. Such results did not occur in experiments used for data analysis. Moreover, such permeability chambers (with membrane tears) did not respond significantly to the test agents.

Diapedesis experiments. BAEC monolayers cultured in permeability chambers were rinsed three times with buffer (DMEM containing 0.45% BSA, pH 7.4) at 37°C, the lower wells were rinsed and filled with 200 μL of buffer, and the side holes were stoppered with paraffin. Test agents dissolved in buffer were applied to the monolayers as 200-μL aliquots, and the permeability chambers were incubated at 37°C in a humidified 95% air/5% CO_2 atmosphere. After 30 minutes, the monolayers were rinsed three times with buffer and, if no further agents were to be applied, 1.25×10^6 PMNs in a 200-μL aliquot of buffer (37°C) was applied to each monolayer and the chambers were again incubated in a 37°C tissue culture incubator. After 30 minutes, the monolayers were rinsed three times, the lower wells were sampled, and the number of PMNs in their 250-μl volume was determined with a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). The numbers of PMNs in the lower wells of experimental groups were then compared with values obtained for concurrently conducted controls (in which the monolayers were treated with buffer alone prior to addition of PMNs) and represented as percentages of control values.

In experiments to determine any direct amine influences on PMN diapedesis, PMNs suspended in DMEM buffer at 2 to 5×10^8 PMNs/mL were treated with NE, 5HT, or histamine for 30 minutes at 25°C. The PMNs were then pelleted at 1,200 g for 4 minutes, resuspended in DMEM buffer without amines at 6.25×10^6 cells/mL, and added to chambers containing monolayers that had not received any amine pretreatments. A diapedesis assay was then conducted as described above.

In addition, acellular amnionic membranes mounted in permeability chambers were directly treated with amines for 30 minutes, and a "diapedesis" assay was then conducted, except that 10^{-7} mol/L of *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) was included in the lower wells as a chemoattractant.

Permeability to EBA. An aqueous solution of 5% BSA and 0.5% Evan's blue dye was incubated at 37°C for 15 minutes. Protein was then precipitated repeatedly with 100% ethanol until no free dye was detectable in the washes. The resulting EBA was air dried and stored at 4°C. Gel electrophoresis under denaturing conditions revealed that EBA was a single blue band that comigrated with BSA standards.

BAEC monolayers cultured in permeability chambers were treated with pharmacological agents as previously described, and a 200-μL aliquot of 0.45% EBA in DMEM (37°C, filtered prior to use) was then applied to each monolayer. After a 30-minute incubation at 37°C, the upper wells were aspirated dry, the monolayers were rinsed three times, and the lower wells' volumes were sampled. These samples were then raised to 1.0 mL with distilled water and read for absorbance at 620 nm in a spectrophotometer. The lower the absorbance, the less EBA present, and therefore the less permeable the monolayer.

PMN adherence. PMN adherence to BAEC monolayers was determined using the method of Zimmerman and Hill.[23] In brief, confluent monolayers cultured in 24-well plates were treated with pharmacological agents for 30 minutes at 37°C and then rinsed with buffer three times. A 500-μL aliquot containing 3.2×10^6 PMNs was added to each well, and the plates were incubated at 37°C for 10 minutes. Supernatants were removed, the monolayers were gently rinsed with counting buffer, and the number of nonadherent PMNs was determined with a Coulter counter. Results are represented as the percentage of applied PMNs remaining adherent to the monolayers.

Statistical analysis. One-way analysis of variance and Duncan's multiple-range test were used to compare groups within experiments.[24]

Reagents. Reagents were obtained from the following sources: DMEM from GIBCO, Grand Island, NY; FBS from Hyclone Labs, Logan, UT; Dil-Ac-LDL from Biomedical Technologies, Cambridge, MA; Dextran T500 from Pharmacia Fine Chemicals, Piscataway, NJ; BSA from Boehringer Mannheim, Indianapolis; phenoxybenzamine, diphenhydramine, and cimetidine (Tagamet) from Smith, Kline and French, Philadelphia; propranolol from Ayerst, New York; and ketanserin as a gift of Janssen Pharmaceutica, Beerse, Belgium; all other reagents were obtained from Sigma Chemical, St Louis.

RESULTS

Under control conditions (30-minute monolayer pretreatment with buffer alone) 1.0×10^5 PMNs (8.0% of the PMNs applied) migrate across a BAEC monolayer and the underlying amnionic membrane within 30 minutes (n = 78, SEM ± 3,494). (All percentages are given as mean values.) In the absence of a monolayer, 1.5×10^4 PMNs (1.2% of the PMNs applied) migrate across the amnionic membrane (n = 10, SEM ± 1,073). These results agree with those of Russo and co-workers,[25] who demonstrated that PMN diapedesis across intact amnion membranes (ie, amnion separated from the chorion but otherwise untreated) is enhanced by a viable epithelial cell monolayer. These researchers also reported that the PMN transit occurs uniformly across the monolayer surface through intercellular junctions and closely mimics diapedesis as observed in vivo during inflammation.

If the length of time PMNs are incubated with BAEC monolayers is varied, a maximum level of PMN diapedesis is reached between 15 and 30 minutes and does not increase up to 60 minutes thereafter (n = 6 for each time point, data not shown). Cramer and colleagues[26] found that PMN diapedesis across epithelial monolayers cultured on micropore filters was maximum within 15 minutes in the absence of a chemoattractant, whereas Meyerick and associates[15] found that diapedesis across pulmonary artery explants was maximal by 60 minutes. Therefore, the present study agrees with these previous reports in that PMN diapedesis in in vitro systems reaches some maximum level, the timing of which probably depends on the nature of the substrate (eg, its thickness).

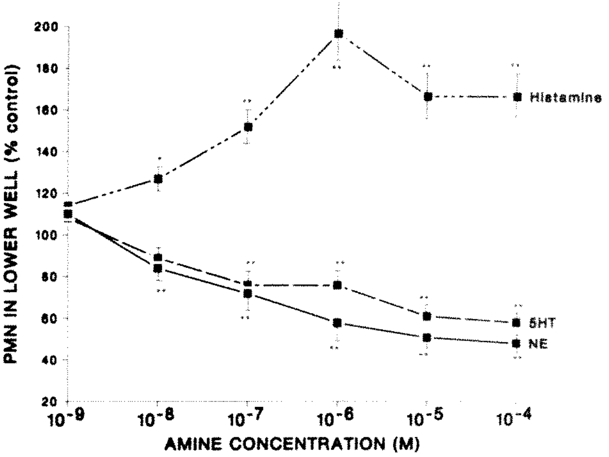

Fig 2. Polymorphonuclear leukocyte (PMN) diapedesis following treatment of bovine aortic endothelial cell (BAEC) monolayers with amines. Significance from controls is noted for $P < .01$ (**) and $P < .05$ (*). Values are represented as means ± SEM, and all are n = 6.

Either NE or 5HT treatment of a monolayer reduces the basal level of PMN diapedesis in a dose-responsive manner (Fig 2). For NE, the minimum significant effect occurs at 10^{-8} mol/L (16% reduction over controls, SEM ± 3%), and the maximum effect occurs between 10^{-6} and 10^{-4} mol/L (58% reduction by 10^{-6} mol/L, SEM ± 6%). For 5HT, the minimum significant effect occurs at 10^{-7} mol/L (24% reduction over controls, SEM ± 4%), and the maximum effect occurs between 10^{-5} and 10^{-4} mol/L (39% reduction by 10^{-5} mol/L, SEM ± 2%). In contrast, histamine increases PMN diapedesis. The minimum significant effect occurs at 10^{-8} mol/L (27% increase over controls, SEM ± 3%), and the maximum effect occurs between 10^{-6} and 10^{-4} mol/L (97% increase by 10^{-6} mol/L, SEM ± 11%).

The addition of the chemoattractant peptide FMLP to the lower well of a permeability chamber significantly increases the number of PMNs migrating across the monolayer, from 1.1×10^5 PMNs (8.8% of the PMNs applied) without FMLP to 1.9×10^5 PMNs (15.2% of the PMNs applied) with FMLP (n = 6). FMLP, however, does not qualitatively alter the amine effects on the BAEC monolayers: NE and

Table 1. Chemoattractant-Stimulated PMN Diapedesis Following Treatment of BAEC Monolayers With Amines

Monolayer Treatment	PMNs in Lower Well (% Control)
Control	100 ± 4
NE	63 ± 4
5HT	53 ± 5
Histamine	121 ± 3

Abbreviations: PMNs, polymorphonuclear leukocytes; BAEC, bovine aortic endothelial cells; NE, norepinephrine; 5HT, serotonin.

A PMN diapedesis experiment was conducted in which *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-7} mol/L) was included in the lower wells of the permeability chambers. Data are expressed as percentages of control values ± SEM. All experimental groups differ significantly from controls ($P < .01$) and are n = 6, and all agents are used at 1×10^{-6} mol/L.

5HT again reduce diapedesis, whereas histamine increases it (Table 1).

If the length of time the monolayers are treated with amines is varied, NE and histamine produce maximal effects between 5 and 15 minutes (Fig 3). In contrast, the maximal effect of 5HT occurs between 15 and 30 minutes. For this reason, 30 minutes was used for treatment incubation times for all other experiments.

When PMNs are first treated with NE, 5HT, or histamine (10^{-6} mol/L) for 30 minutes at 25°C, and then used in diapedesis experiments with untreated BAEC monolayers, no changes in diapedesis are observed as compared with buffer-treated PMNs (n = 12, data not shown). Moreover, when acellular amnionic membranes (without a BAEC monolayer) are directly treated with amines (10^{-6} mol/L), no changes in PMN chemotaxis across the membranes are observed as compared with controls (n = 6, data not shown).

When a BAEC monolayer is treated with the α-adrenergic antagonist phenoxybenzamine or the β-adrenergic antagonist propranolol and is then treated with both NE and the same receptor antagonist, the expected NE effect (a decrease in diapedesis) does not occur (Table 2). In contrast, treatment with the 5HT S_2 receptor antagonist ketanserin prior to ketanserin/5HT cotreatment does not significantly alter the expected 5HT effect of reduced diapedesis.

The histamine H_1 receptor antagonist diphenhydramine, when used as a pretreatment to diphenhydramine/histamine cotreatment, completely blocks the expected effect of increased diapedesis (Table 2). In contrast, cimetidine, a histamine H_2 receptor antagonist, does not block the histamine effect but slightly potentiates it.

Table 2. PMN Diapedesis Following Treatment of BAEC Monolayers With Amines or Amine Receptor Antagonists/Amines

Monolayer Treatment	PMNs in Lower Well (% Control)
Control	100 ± 3
NE	66 ± 2
Phenoxybenzamine/NE	107 ± 5
Propranolol/NE	107 ± 5
5HT	70 ± 3
Ketanserin/5HT	61 ± 3
Histamine	140 ± 4
Diphenhydramine/histamine	97 ± 4
Cimetidine/histamine	165 ± 3

Monolayers were pretreated for 30 minutes with a receptor antagonist followed by a 30-minute cotreatment with the same antagonist and the appropriately corresponding amine, or with buffer alone for 30 minutes followed by an amine for 30 minutes, or with buffer alone for 60 minutes (control). Data are expressed as percentages of control values ± SEM. NE, 5HT, ketanserin/5HT, histamine, and cimetidine/histamine differed significantly from controls ($P < .01$), and cimetidine/histamine differed significantly from histamine alone ($P < .01$). All agents were used at 1×10^{-7} mol/L, and all groups were n = 6.

Monolayers were also treated with two agents known to alter actin microfilaments directly, as a means of assessing any EC cytoskeletal influence on diapedesis. Phalloidin, which polymerizes G-actin to F-actin microfilaments and stabilizes these filaments,[27] decreases diapedesis in a dose-responsive manner (Fig 4). The minimum significant effect occurs at 10^{-10} mol/L (21% reduction over controls, SEM ± 5%), and the maximum effect occurs between 10^{-9} and 10^{-8} mol/L (67% reduction over controls by 10^{-9} mol/L, SEM ± 2%). In contrast, cytochalasin B, which inhibits G-actin polymerization,[28] increases diapedesis. The minimum effect occurs at 10^{-7} mol/L (51% increase over controls, SEM ± 7%), and the maximum effect occurs between 10^{-5} and 10^{-4} mol/L (128% increase over controls by 10^{-5} mol/L, SEM ± 7%).

When monolayers are treated with either NE, 5HT, or

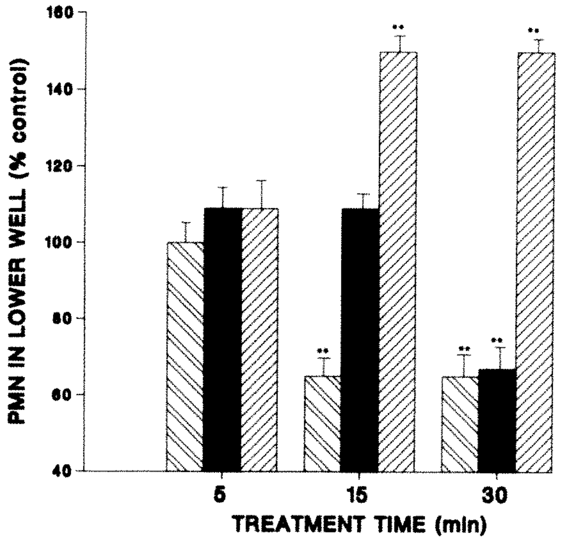

Fig 3. Polymorphonuclear leukocyte (PMN) diapedesis following treatment of bovine aortic endothelial cell (BAEC) monolayers with amines for varying times. All amines are used at 1×10^{-7} mol/L. Significance from controls is noted for $P < .01$ (**). Values are represented as means ± SEM, and all are n = 6. ▧, NE; ■, 5HT; ▨, histamine.

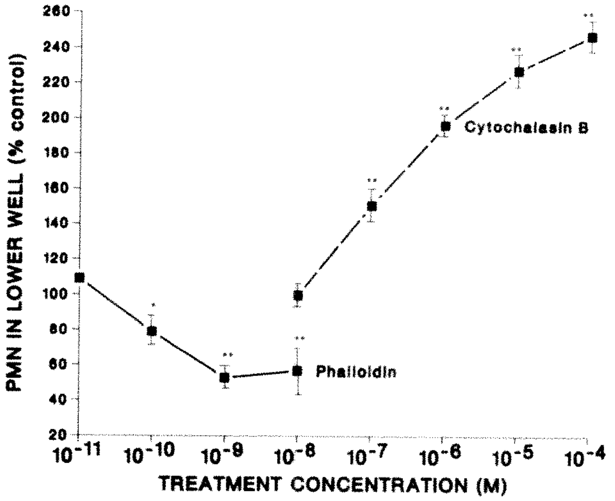

Fig 4. Polymorphonuclear leukocyte (PMN) diapedesis following treatment of bovine aortic endothelial cell (BAEC) monolayers with phalloidin or cytochalasin B. Significance from controls is noted for $P < .01$ (**) and $P < .05$ (*). Values are represented as means ± SEM, and all are n = 6.

histamine for 30 minutes and then with buffer alone for 30 minutes, no significant effects on PMN diapedesis are evident as compared with controls (ie, treatment with buffer alone for 60 minutes) (Fig 5). This demonstrates the reversibility of the amines' effects. Phalloidin's effect is also reversible within 30 minutes; the effect of cytochalasin B, however, is only partially reversed 30 minutes posttreatment (to 65% of the effect at 0 minutes posttreatment, SEM ± 6%).

Monolayer permeability to EBA is similarly affected by amine, receptor antagonist/amine, phalloidin, or cytochalasin B treatments (Table 3). NE reduces permeability, with the effect being completely blocked by propranolol and phenoxybenzamine; 5HT likewise reduces permeability, but its action is unaffected by ketanserin. Histamine increases permeability, and diphenhydramine completely blocks the effect. Finally, cytochalasin B increases permeability, whereas phalloidin decreases it.

PMN adhesion to BAEC monolayers is unaffected by previous monolayer treatment with NE, 5HT, histamine, cytochalasin B, or phalloidin (Table 4). The concentrations used significantly affect diapedesis, however.

DISCUSSION

These experiments demonstrate that specific vasoactive amines modulate the level of PMN diapedesis across a BAEC monolayer in vitro. The amines NE and 5HT significantly reduce diapedesis; histamine, conversely, increases diapedesis. These changes can be attributed to direct amine effects on the ECs, because amine treatment of EC produces the diapedesis changes whereas amine treatment of PMN does not affect their subsequent diapedesis across untreated monolayers. Also, amine treatment of amnionic membranes (without a BAEC monolayer) does not affect PMN chemotaxis across the membranes. This conclusion is supported in part by Clark and colleagues,[29] who also reported no effect of histamine on PMN migration. Therefore, BAEC are capable of presenting a greater or lesser barrier to PMN diapedesis, depending on exposure to exogenous amines.

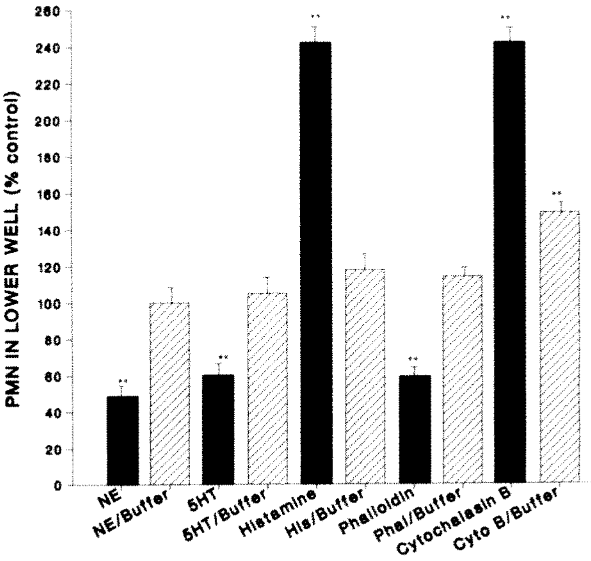

Fig 5. Reversal of the amine, phalloidin, and cytochalasin B effects on polymorphonuclear leukocyte (PMN) diapedesis. His, histamine; Phal, phalloidin; and Cyto B, cytochalasin B. Solid bars, 30-minute treatment with agents; striped bars, 30-minute treatment with agent followed by a 30-minute treatment with buffer alone. Significance from controls is noted for $P < .01$ (**). In addition, cytochalasin B/buffer significantly differs from cytochalasin B alone ($P < .01$). All treatments are 1 × 10^{-6} mol/L, except for phalloidin (1 × 10^{-8} mol/L). Values are represented as mean ± SEM, and all are n = 6.

Table 3. BAEC Monolayer Permeability to EBA Following Treatment With Various Agents

Monolayer Treatment	Absorbance (% Control)
Control	100 ± 2
NE	69 ± 4
Phenoxybenzamine/NE	92 ± 4
Propranolol/NE	102 ± 2
5HT	56 ± 3
Ketanserin/5HT	60 ± 4
Histamine	121 ± 2
Diphenhydramine/histamine	94 ± 3
Phalloidin	32 ± 3
Cytochalasin B	123 ± 4

Abbreviation: EBA, Evan's blue-labeled albumin.

Monolayers were treated with amine receptor antagonists and amines as previously described (Table 2); phalloidin and cytochalasin B treatments were for 30 minutes. Data are expressed as percentages of control values for absorbance ± SEM. NE, 5HT, ketanserin/5HT, histamine, phalloidin, and cytochalasin B differed significantly from control ($P <$ .01). All agents were used at 1 × 10^{-6} mol/L, except for phalloidin (1 × 10^{-8} mol/L); all groups were n = 6.

Unless otherwise stated, the permeability chambers contained buffer alone in their lower wells. Addition of a PMN chemoattractant (10^{-7} mol/L of FMLP) to the lower wells, however, does not qualitatively alter the amine effects on the BAEC monolayers. This further supports the relevance of the present work to PMN diapedesis during inflammatory states.

The amine-induced changes in diapedesis are reversible. A 30-minute monolayer incubation with buffer alone reverses any previous amine treatment, and PMN diapedesis returns to control values. Moreover, none of the amine treatments used produced gross morphological changes as visible with light microscopy, and none of the amine or other treatments used decreased EC viability (as assessed by trypan blue and

Table 4. PMN Adhesion to BAEC Monolayers Following Treatment of Monolayers With Various Agents

Monolayer Treatment	Adherent PMNs (% Applied)
Control	13 ± 2
NE	11 ± 1
5HT	11 ± 1
Histamine	12 ± 1
Phalloidin	13 ± 1
Cytochalasin B	12 ± 1

Data are expressed as the percentage of applied PMNs remaining adherent to the monolayers following a 10-minute incubation, ± SEM. Monolayers had been previously treated with amines or cytochalasin B (1 × 10^{-7} mol/L) or phalloidin (1 × 10^{-8} mol/L) for 30 minutes; all groups were n = 6.

fluorescein diacetate/ethidium bromide vital staining). Moreover, the amine concentrations used were comparable to levels ranging from normal conditions[30-32] to those of thrombogenesis. These observations support our thesis that vasoactive amines alter EC physiology and that regulation of barrier function may occur in vivo, as we have previously suggested for monolayer permeability to macromolecules and the erythrocyte extravasation associated with severe thrombocytopenia.[6,7]

β-adrenergic receptors on BAEC has been demonstrated using a radiolabeled ligand.[33] In the present study, the effects of NE on diapedesis and albumin permeability were blocked by both phenoxybenzamine and propranolol, suggesting that the amine may act through adrenergic receptors.

Histamine H_1 receptors have also been localized on aortic EC using a radiolabeled ligand.[34] In the present study, histamine-induced diapedesis and permeability increases were completely blocked by diphenhydramine, suggesting that they may be due to H_1 receptor stimulation. Cimetidine's potentiation of histamine's effect on diapedesis may be due in part to some H_1 receptor agonistic activity or to a nonreceptor-mediated action.

The 5HT-induced diapedesis and permeability alterations appear to be nonreceptor mediated, in that the 5HT S_2 receptor antagonist ketanserin does not block the amine's effects. This finding supports Makarski's report of the lack of 5HT receptors on cultured BAEC,[35] a conclusion also drawn in our own work using radiolabeled ligand localization[36] and receptor binding and pulse-chase experiments. The longer time necessary for the appearance of a maximal 5HT effect on diapedesis, as compared with the time necessary for maximal NE and histamine effects, may reflect the nonreceptor-mediated action of 5HT *v* the receptor-mediated action of the other two amines.

Histamine causes a transient dilation of EC junctions,[18,37,38] leading to increased permeability to water and sucrose[18] and albumin.[6,38] In contrast, NE and 5HT decrease EC monolayer permeability to albumin[6] and prevent the erythrocyte extravasation associated with thrombocytopenia in vivo,[7] effects believed to reflect increased EC junctional integrity. Increased monolayer permeability as a result of decreased junctional integrity has been attributed to alterations in the EC cytoskeleton,[10-13] and Meza and colleagues[39] related alterations in microfilament patterns to changes in epithelial cell monolayer electrical resistance, an indicator of junctional integrity. Direct manipulation of microfilaments with cytochalasin B also decreases monolayer junctional integrity[10,14,39] and increases permeability to albumin,[10] further establishing the connection among cytoskeletal arrangements, junctional integrity, and monolayer permeability.

Previously we showed that NE and 5HT increase, whereas histamine decreases, EC stress fiber numbers.[8] We also showed that NE and 5HT act directly to increase G-actin to F-actin polymerization, as determined by G-actin inhibition of DNase I activity.[39a] We believe that the results reported here on amine-induced alterations in PMN diapedesis and monolayer permeability to albumin may be due to these actions: Amine-induced EC cytoskeletal alterations may alter monolayer junctional integrity as described above, leading to both diapedesis and permeability changes. Because PMN diapedesis occurs intercellularly,[1,15,25] as does the bulk of trypan blue-labeled albumin transport across BAEC monolayers,[40] changes in monolayer junctional integrity would be expected to alter both PMN diapedesis and albumin permeability. The effects of phalloidin and cytochalasin B further support this conclusion: Stabilization of EC microfilaments by phalloidin decreases, whereas disruption of microfilaments by cytochalasin B increases, diapedesis and permeability. In addition, as with the amines, the effects of phalloidin and cytochalasin B are dose-responsive and reversible, which may further suggest a commonality of action between these agents and the amines. Wong and Gottlieb[14] report a time course and effective concentration range for the disruption by cytochalasin B of the dense peripheral band of microfilaments in ECs similar to the time course in the present report for the agent's effects on diapedesis (ie, 10^{-6} mol/L is effective within 15 minutes of treatment, and the effect is reversible between 15 and 60 minutes following removal of the treatment).

Other mechanisms could contribute to the amine effects on EC barrier function. Because PMN adhesion to the endothelium occurs prior to diapedesis, alterations in PMN adhesion could increase or decrease diapedesis. Such a mechanism does not appear to be involved in the present work, however, as neither the amines, cytochalasin B, nor phalloidin affected PMN adhesion to BAEC monolayers at concentrations that affected diapedesis. Several reports have also suggested that ECs can elaborate factors capable of influencing PMN-EC interactions.[41-43] Such factors would not be expected to alter both diapedesis and monolayer permeability to albumin, however, as evidenced in this study. We found that BAECs do release factor(s) that influence PMN diapedesis and that this release is influenced by NE and 5HT (J. Doukas, D. Shepro, H.B. Hechtman, unpublished observations). We are now examining the relationship between the release of these factors and the amine-induced cytoskeletal alterations.

In conclusion, we present experimental evidence that NE or 5HT treatment of a BAEC monolayer reduces subsequent PMN diapedesis across the monolayer whereas histamine increases subsequent diapedesis. These effects are suggested to reflect amine-induced alterations in the EC cytoskeleton.

ACKNOWLEDGMENT

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In Vivo Induction of Proteins During Therapy of Hairy Cell Leukemia With Alpha-Interferon

By Brian L. Samuels, Harvey M. Golomb, and Bernard H. Brownstein

The mechanism of the antineoplastic effect of interferon (IFN) is not known and may result from direct effects on the neoplastic cells themselves, activation of intermediary effector cells, or a combination of these effects. The synthesis of specific proteins is induced in hairy cells when they are exposed to α-IFN in vitro. In particular, the synthesis of an 80,000-dalton protein, which we have called p80, is markedly induced. We investigated this effect in the hairy cells of seven patients in the leukemic phase of hairy cell leukemia who were being treated with subcutaneous (SC) IFN α2b (r-Hu-IFN-α2). Polyacrylamide gel electrophoresis (PAGE) was carried out on [^{35}S]methionine-labeled whole cell lysates visualized by autoradiography and silver staining. Within 2 days of starting IFN therapy, induction of specific protein synthesis, including p80, was seen by [^{35}S]methionine labeling in freshly isolated circulating hairy cells from 6 of 6 patients tested. Therefore, α-IFN has a direct biochemical effect on hairy cells in vivo that is similar to in vitro effects, at least with regard to p80 synthesis, although the kinetics of this effect may vary in the two situations.

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THE USE OF biological response modifiers is a promising new field in the therapy of malignant disease. One of the most successful applications has been the treatment of hairy cell leukemia (HCL) with α-interferon (α-IFN).[1-3] The mechanism of the antineoplastic effect of IFN remains unknown, despite extensive investigation of the problem. We investigated the direct biochemical effects of IFN on hairy cells and showed that hairy cells in culture respond to exogenous recombinant α-interferon (r-Hu-IFN-α2) by the induction of synthesis of specific proteins.[4] For some time, IFN has been known to induce the synthesis of specific proteins in cells exposed in vitro.[5-7] Hairy cells, however, are particularly sensitive to very low IFN concentrations; in addition, more proteins are induced, and for a far longer period than previously reported in other cell types.[4] Most patients with HCL are pancytopenic, and bone marrow fibrosis usually precludes marrow aspiration.[8] For these reasons, it is difficult to obtain samples of hairy cells from patients for in vitro experimentation. A few patients with HCL develop a leukemic phase during their illness, however, during which phase leukocyte counts are >10,000/μL, and >50% of the WBCs are hairy cells. From the large referral base of HCL patients at our institution, we were able to select several such patients who were about to begin therapy with α-IFN. Using samples from these patients, we investigated the in vivo induction of specific proteins in hairy cells by pharmacological doses of r-Hu-IFN-α2.

MATERIALS AND METHODS

Materials

r-Hu-IFN-α2 was kindly provided by Schering Corporation (Bloomfield, NJ). [^{35}S]Methionine 15 Ci/mmol was from Amersham (Arlington Heights, IL). EN^{3}HANCE fluorographic solution was purchased from New England Nuclear Corporation (Boston). All other reagents were from commercial sources and were of reagent grade.

Methods

Peripheral blood samples were obtained from seven patients with HCL prior to the initiation of IFN-α-2b therapy. All patients were in the leukemic phase of the disease (Table 1). Additional samples were obtained at 2 days (6 patients), and 2 (2 patients) or 4 weeks (6 patients) after IFN therapy was started. Mononuclear cells were isolated on a Ficoll-Hypaque gradient,[9] and brought to a concentration of 2 × 10^6 cells/mL. All patients were advised of the procedures and possible risks, according to institutional guidelines, and all gave informed consent.

Pre-IFN samples. r-Hu-IFN-α2 was added to flasks of the cell suspension at 1,000 IU/mL. Similar flasks without added IFN were used as controls. The flasks were incubated for 24 hours at 37°C in 5% CO_2, after which the cells were transferred to methionine-poor RPMI 1640 Select-Amine medium (GIBCO, Grand Island, NY). [^{35}S]Methionine at 100 μCi/mL was added to each flask. Cells were pulse-labeled for 4 hours and lysed according to the method of Laemmli,[10] with protease inhibitors added to the lysis buffer. Nucleic acids were sheared by serial passage through syringe needles down to 30 gauge. Samples were stored at −80°C until used.

IFN therapy. The patients were subsequently started on therapy with r-Hu-IFN-α2, at a dose of 2 × 10^6 IU/m^2 subcutaneously (SC) three times weekly for 1 year.

Duration of induction. Flasks of pre-IFN hairy cells were used in these experiments. One set of flasks had no IFN added. The other flasks had r-Hu-IFN-α2 at 1,000 IU/mL added as above. After 24 hour incubation with added IFN, cells from two sets of flasks were washed several times and resuspended in IFN-free medium. At 4 and 9 days, respectively, after being washed free of IFN, cells from these two sets of flasks were transferred to methionine-poor Select-Amine and pulse-labeled with [^{35}S]methionine as described above. Another set of flasks was incubated with added IFN for 9 days before pulse-labeling. The cells that had been incubated without IFN for 9 days were transferred to "spent" medium (prepared by incubating hairy cells in IFN-containing medium for 9 days and then centrifuging to remove the hairy cells, leaving a cell-free spent medium). After 24-hour incubation in the spent medium, the cells were transferred to Select-Amine and pulse-labeled as described above.

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Table 1. Patient Peripheral WBC Counts and Percentage of Hairy Cells Before and After IFN Therapy

	Day 0 of IFN Therapy		Day 28 of IFN Therapy	
Patient	WBC Count (Cells/μL)	Hairy Cells (% of MNC)	WBC Count (Cells/μL)	Hairy Cells (% of MNC)
R.H.	60,500	95	5,200	28
H.B.	47,500	75	7,800	15
L.P.*	24,600	62†	10,100	36
C.T.	48,800	93	10,200	67
C.O.	23,300	79	7,300	26
G.H.*	16,000	79	2,500	—‡
W.M.	34,600	90	9,100	73

*Two-week samples obtained; data for hairy cell proportion not available.

†Most mononuclear cells not classified as hairy cells on peripheral smear stained positive for tartrate-resistant acid phosphatase, giving a probable higher actual hairy cell proportion in this patient.

‡No 4-week sample obtained.

Electrophoresis. Polyacrylamide gels were prepared by a modification of the method of Laemmli,[10] whereby the acrylamide:methylene *bis*-acrylamide ratio was 30%:0.1%. Separating gels of 12% polyacrylamide were used. Electrophoresis was carried out at a constant 200 V. Gels were fixed in 30% methanol:10% acetic acid for 1 hour, impregnated with EN^{3}HANCE, dried under vacuum, and exposed to Kodak XAR-5 film at −80°C for 1 to 10 days.

Post-IFN samples. Mononuclear cells were isolated from peripheral blood as described above and placed directly into methionine-poor Select-Amine at 2×10^6 cells/mL. [^{35}S]Methionine was added at 100 μCi/mL, and the cells were pulse labeled for 4 hours. Whole cell lysates were prepared as described above, and electrophoresis and autoradiography were done. Samples from one patient were not radiolabeled; therefore, after electrophoresis, the protein bands were visualized by silver stain only,[11] and the gels were photographed. Radiolabeled cell lysates from two of the other patients were analyzed after electrophoresis by silver staining as well as by autoradiography.

RESULTS

Exposure in vitro to (r-Hu-IFN-α2) resulted in induction of synthesis of several proteins in hairy cells, as analyzed by one-dimensional polyacrylamide gel electrophoresis (PAGE) (Fig 1). The most prominent induced protein was ~80,000 daltons, and was designated p80. Lysates prepared from hairy cells obtained from six patients 2 days after initiation of continuing r-Hu-IFN-α2 therapy also showed induction of p80 synthesis, without addition of IFN during the [^{35}S]methionine labeling period in vitro (Fig 1). The *only* IFN exposure that these circulating hairy cells received occurred in vivo, as a result of r-Hu-IFN-α2 therapy. In gels of cell lysates obtained from two of these patients after 2 weeks of therapy, and from five of the patients examined after 4 weeks of therapy, induction of p80 synthesis was not detectable, either by silver stain or by autoradiography. Samples from patient W.M. were analyzed by silver stain only, and did show induction of p80 after 4 weeks of IFN therapy.

When hairy cells were incubated with IFN for 9 days, induction of p80 synthesis was still taking place at 9 days (Fig 2). Previously unexposed hairy cells incubated in IFN-containing spent medium (prepared by incubating hairy cells in IFN-containing medium for 9 days, then centrifuging to remove the hairy cells, leaving a cell-free spent medium), were also induced to synthesize p80 (Fig 2). Thus, hairy cells were still capable of being induced to synthesize p80 after 9 days in culture, and the IFN remained biologically active after 9 days in contact with hairy cells in the medium. Hairy cells incubated in IFN-containing medium for 24 hours and then resuspended in IFN-free medium showed diminished induction of p80 synthesis at 4 days and 9 days after resuspension (Fig 2).

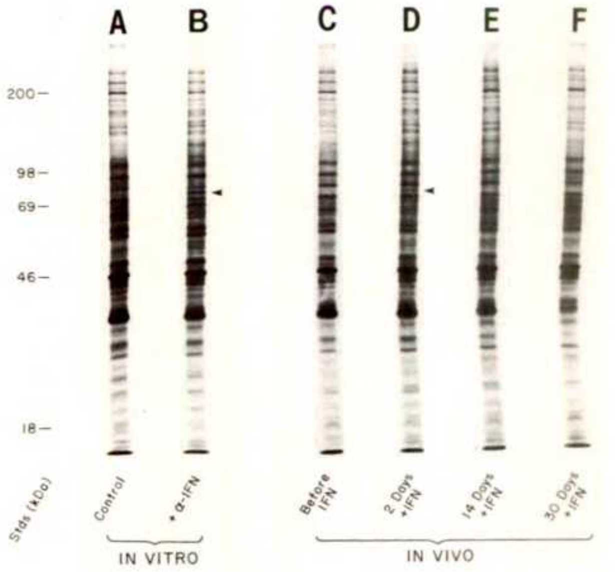

Fig 1. Autoradiogram showing induction of the p80 protein in hairy cells by α-interferon (α-IFN) in vitro and in vivo. Lane A, hairy cells incubated in IFN-free medium; lane B, cells incubated in vitro with IFN for 24 hours prior to labeling; lane C, cells isolated immediately prior to initiation of IFN therapy; lanes D through F, cells isolated at 2, 14, and 30 days after the initiation of therapy, respectively. Several α-IFN–induced proteins are evident in lane B; arrow marks position of p80, the most prominently induced protein.

DISCUSSION

The data show that the previously noted induction of synthesis of specific proteins in hairy cells by (r-Hu-IFN-α2) in vitro[4] also occurs in vivo in the cells of patients being treated with this IFN. We believe that the data imply that induction of synthesis occurs in hairy cells since in three of the patients hairy cells constituted 90% to 95% of the circulating mononuclear cells. In support of this interpretation is the fact that when the proportion of circulating hairy cells was reduced by therapy, in most cases to <50%, the induction was no longer detected, although a similar number of mononuclear cells were tested. In vivo induction of protein synthesis in the cells of patients on therapy with IFN has previously been demonstrated in terms of 2′-5′ oligoadenylate synthetase activity in circulating mononuclear cells of such patients.[12] Induction of protein synthesis to levels detectable by PAGE has not previously been reported in vivo, and this illustrates the quantitative significance of the amount of p80 induced in the circulating hairy cells of the patients whom we studied. To our knowledge, this is the first description of a

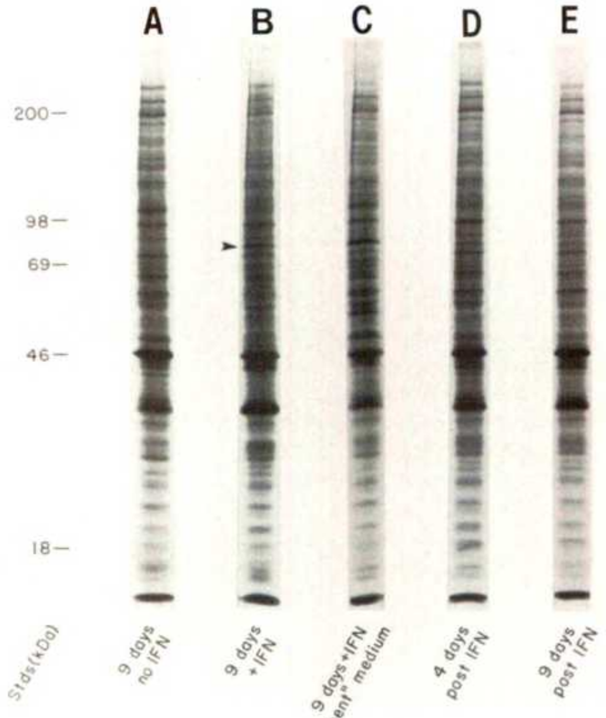

Fig 2. Autoradiogram of whole cell lysates (12% gel) from cells exposed to interferon (IFN) and then resuspended without IFN for various time periods. Lanes A and B, experimental control: lane A, cells incubated in IFN-free medium and lane B, cells incubated in medium with IFN for 24 hours prior to labeling. Lane C shows unexposed hairy cells suspended in a medium that initially contained IFN and hairy cells for 9 days. The "spent" medium was cleared of cells prior to the addition of the unexposed cells, and then incubated for 24 hours. For lanes D and E, hairy cells were exposed to IFN for 24 hours in vitro, washed three times, and then incubated in IFN-free medium for the indicated number of days before the lysate was made (described in the Materials and Methods section). Arrow marks position of the p80.

biochemical effect of IFN directly on human neoplastic cells in vivo.

In parallel with our findings from in vitro investigation of the induction phenomenon, the in vivo induction of synthesis of p80 occurred rapidly and was observed within 2 days of the initiation of therapy. Previously, we showed that induction of p80 synthesis in vitro occurred at IFN concentrations ranging from 0.1 to 10,000 IU/mL. At the dose of r-Hu-IFN-α2 used clinically (2×10^6 IU/m^2 SC), a serum IFN level of ~100 to 200 IU/mL would be attained at ~4 hours, with elimination over the next 12 hours.[13]

The current data show that, in vitro, induction of synthesis will continue for at least 9 days in the continued presence of IFN, but that induction is diminished after 4 days without exogenous IFN and remains at this level even at 9 days without IFN. In vivo, the interaction of drug elimination and cell turnover might well lead to a more rapid decline in the induction response, so that it is not at a level detectable by PAGE after 2 weeks of IFN therapy. Regulation of the induction of protein synthesis may differ between hairy cells in vivo and in vitro at either a transcriptional, a translational, or a posttranslational level. Down-regulation of the IFN receptor may also occur at different rates in the two situations. The kinetics of induction in vivo may change with time and with turnover of the circulating hairy cell population, so that there is a more rapid decay of the mRNA transcribed and/or a more rapid degradation of the p80 protein (since it was not observed to be induced on silver stain visualization, as well as the autoradiograms). The induction seen by silver stain in one patient (W.M.) after 4 weeks of therapy may indicate some patient-to-patient variability in the parameters of the IFN response in vivo. In addition, the proportion of mononuclear cells that were hairy cells had greatly diminished by 4 weeks of IFN therapy (Table 1). (Patient W.M. had the highest residual proportion of hairy cells at 4 weeks, 73%.)

The antitumor effect of IFN remains unexplained. The effect may be mediated indirectly by interaction with intermediary cells such as natural killer (NK) cells, which then act against neoplastic cells.[14] IFN may also have a direct antiproliferative effect on the tumor cells. Alternatively, both mechanisms may play a role. Recent data[15] support older reports[16] that neither NK activity nor an intact immune system is an absolute requirement for inhibition of tumor growth by IFN in vivo in animal models.

IFN therapy does lead to normalization of NK activity,[17] which is characteristically low in patients with HCL.[18] This phenomenon, however, appears to accompany rather than precede the return of all peripheral blood cell counts toward normal. Our data certainly indicate that there is a direct, measurable biochemical effect of IFN on the target hairy cells in vivo when patients are treated with r-Hu-IFN-α2. Whether induction of synthesis of specific proteins such as p80 is involved in the antineoplastic effect of IFN is not known, but it is a marker of direct effects of IFN on a neoplastic cell population, and the extreme sensitivity of hairy cells to IFN in terms of induction of specific protein synthesis, as compared with other cell types, parallels the clinical sensitivity of hairy cell leukemia to IFN therapy.

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Differences in Nonspecific Esterase from Normal and Leukemic Monocytes

By Perry D. Cohn, Peter D. Emanuel, and Marek J. Bozdech

Nonspecific esterase (NSE) is used to identify normal and leukemic mononuclear phagocytes cytochemically. It can be demonstrated by the hydrolysis of α-naphthyl acetate or butyrate. NSE from leukapheresed leukemic monocytes were extracted with several types of detergent and grouped into two isoelectric point (pI) categories based on the ease of solubility: pI 5.7-6.3 (8 bands) $\geq$pI 6.6-7.6 (6 bands). Normal monocytes yielded only the pI 5.7-6.3 isozymes. Isozymes from both leukemic and normal monocytes were inhibited similarly by sodium fluoride, pH $<$ 4.7, and a serine active site inhibitor. All isozymes were bound by Sepharose-concanavalin A (Con-A) and displayed similar substrate preferences and pH *v* activity slopes. Under the conditions of detergent solubilization, the smallest mol wt species retaining enzymatic activity was 50 ± 5 kd. Despite these similarities, the isozymes of pI 5.7 through 6.3 and pI $>$ 6.3 exhibited different degrees or types of inhibition by phenylmethylsulfonyl fluoride (PMSF), resistance to heat and antigenic character. Thus, the esterase isozymes represent two families of glycoproteins, both of them probable cell surface enzymes, resembling classical liver carboxyl or B-esterases (EC 3.1.1 1).

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NONSPECIFIC esterases (NSEs) are widely distributed in many types of cells. They are demonstrated and assayed by the cleavage of a short-chain acyl group from a chromogenic leaving group. Their actual function is poorly understood, however, although a list of liver esterase substrates, carboxylic acid esters, has been assembled.[1] Esterase cytochemistry is commonly used to differentiate various types of leukocytes. Mononuclear phagocytes are the most proficient at cleaving α-naphthyl acetate/butyrate substrates, displaying cell surface[2,3] and granular[3,4] activities. Several types of lymphocytes also possess an esterase activity, including a "punctate" form.[2] Since Bozdech and Bainton[2] demonstrated the plasmalemmal location of monocytic NSE, it is clear that in monocytes NSE is not a lysosomal hydrolase. On the cell surface, NSE may have a role in relating to the extracellular environment or to other leukocytes. For instance, Kitahara and Eyre[5] observed a correlation between NSE activity and chemotaxis.

Electrophoresis and isoelectric focusing (IEF) of detergent extractions of whole cell lysates[6-9] or of lysosomes[10] have been used to compare the different groupings of esterase isozymes in various types of blood cells. Our studies have stressed the extraction of ectozymes by brief exposure of live leukapheresed leukemic monocytes to detergent as well as extraction of freeze-thaw lysates or stronger and repeated detergent extractions.

We separated two families of NSEs from leukemic monocytes in agreement with Scott and colleagues.[9] One group is similar to that found in normal monocytes. Both are 50-kd glycoproteins that are sensitive to pH 4.5, NaF, and the serine esterase inhibitor phenylmethylsulfonylfluoride (PMSF). They differ, however, with regard to ease of detergent extraction, resistance to elevated temperatures, and precipitation with antisera.

MATERIALS AND METHODS

Cells. Two patients with acute monocytic leukemia (French-American-British [FAB] classification M5) were therapeutically leukapheresed to reduce their dangerously high leukocyte counts. Pheresed leukocytes from both patients were >85% α-naphthyl acetate or α-naphthyl butyrate nonpunctate esterase positive (ie, mononuclear phagocytes, not T cells), using the method of Li and colleagues[6] or Bozdech and Bainton[2] (described below). As judged by Wright's staining, the leukapheresed mononuclear phagocytes from both patients were predominantly monoblasts. They were negative for peroxidase and weakly positive for acid phosphatase. Equal numbers of leukapheresed cells from both patients were positively stained by antimonocyte .1 monoclonal antibody (Bethesda Research Laboratories, Gaithersberg, MD) but not by OKT3 (Ortho Pharmaceutical, Westwood, MA). Based on Wright's and chloroacetate esterase staining, no platelets and only 5% each of neutrophils and lymphocytes were present. Electron cytochemistry of the esterase was performed using the methods of Bozdech and Bainton.[2] NSE was localized both on the cell surface and intracellularly, as judged by thick and thin section (Figs 1 and 2).

Normal monocytes were obtained from normal volunteers and purified by Ficoll-Hypaque centrifugation. Further purification (up to 90% to 95% monocytes) was effected by reversible adherence to fibronectin-coated plastic Petri dishes.[11] Purified fibronectin was supplied by Deane F. Mosher. Iron carbonyl separation of monocytes was performed in some experiments.

Enzyme extraction. Leukapheresed leukemic monocytes were cleared of RBCs by 0.83% NH_4Cl lysis. The cells were briefly vortexed with equal volumes of detergent solution [0.1% cetyltrimethylammoniumbromide (CTAB) or 0.1% to 1.0% Triton X-100 or 0.1% 3-3 cholamidopropyldimethylammonio-1-propanesulfonate (CHAPS)] in phosphate-buffered saline (PBS) followed by centrifugation at 2,000 g. Following a 60-second extraction, the sedimented cells appeared intact by electron microscopy except for loss of the plasmalemmal membrane. No enzyme inhibitors were included because the inhibitor specificities were not yet established. The supernatant was centrifuged a second time and frozen. Thawing was accompanied by an additional centrifugation, followed in some experiments by ultrafiltration through a 0.2-μm filter.

Isoelectric focusing. Analytical polyacrylamide IEF was conducted in 5% gels incorporating 1% Triton X-100 and pH 3-10 ampholines enriched with pH 5-7 or 5-8 ampholines. Bands containing NSE activity were stained with the same techniques as used for the cells.[2]

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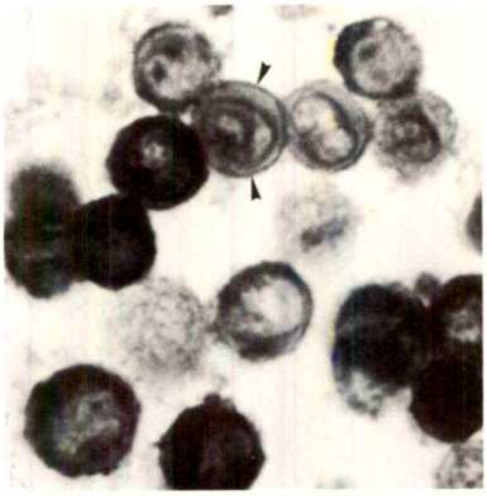

Fig 1. Plastic embedded section of leukapheresed cells from patient 2. Leukemic mononuclear phagocytes exhibit nonspecific esterase (NSE) by colored reaction product both intracellularly and on the cell surface (arrow heads). Original magnification ×900; current magnification ×450.

Measurement of NSE. NSE was measured by the techniques of Bozdech and Bainton,[2] which were adapted from Li and colleagues.[6] This method was dependent on the hydrolysis of α-naphthyl acetate or butyrate (Sigma) in Tris or phosphate buffer (pH 7.2) followed by the reaction of the α-naphthyl leaving group with the hexazotized dye pararosaniline, producing a red color that can be monitored at 500 nm. The esterase activity was either estimated microscopically (cells), judged grossly (polyacrylamide gels), or quantitated by spectrophotometry (enzyme purification). The reaction was stopped at 1 minute with pH 4.5 buffer for spectrophotometry.

Affinity chromatography purification. Sepharose-4B beads conjugated with concanavalin A (Con A, Sigma) were washed and added to the detergent extract. Titration determined that a small amount of beads could bind all NSE activity from the detergent suspension. Based on that titration, Sepharose-Con A was used to purify the extract partially. After extensive washing in buffer, 50 mmol/L of mannose in pH7 PBS was used to elute the NSE from the beads. The NSE activity and Coomassie R-250 (Bradford assay) estimated protein concentration were measured at each step. The amount of protein in the eluate was 5% of that in the initial extract, whereas 70% to 80% of the enzyme activity was recovered. Analytical IEF demonstrated that all original NSE bands were present in the eluate. The eluate was dialyzed in PBS and either concentrated by lyophilization or by concentrative ultrafiltration techniques using Nuclepore CX-10 filters (Nuclepure Corp, Pleasanton, CA).

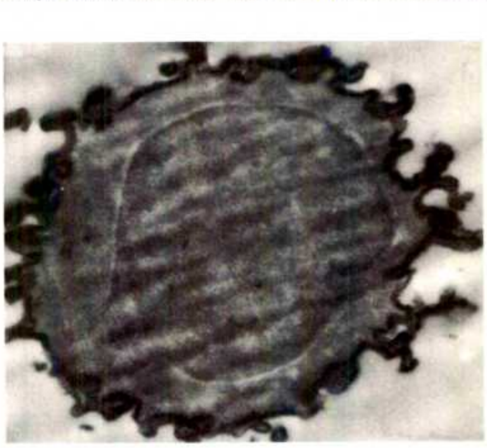

Fig 2. Electron micrograph of a leukapheresed cell from patient 1 exhibiting cell surface NSE staining. Original magnification ×4,500; current magnification ×2,250.

Preparative IEF. The preparative IEF purfication of NSE used granulated, low electroendosmotic polyacrylamide (Biogel; Biorad, Richmond, CA) or 0.5% agarose/4% polyacrylamide gels incorporating a mixture of pH 3-10 and pH 5-8 ampholines. Crude detergent extract or extract partially purified on Sepharose-Con A, dialyzed in 2% tricine-glycine or 1% ampholytes was placed on the prefocused gels. After the run, the NSE activity was localized by blotting with nitrocellulose paper. That area of the bed was removed, and the NSE was eluted with PBS. This method succeeded in purifying the NSE 50-fold, as judged by the change in the ratio of enzyme activity to protein concentration. The eluate was concentrated by ultrafiltration. Up to several hundred-fold purification, as judged by Coomassie staining of analytical IEF gels, was accomplished by combining Sepharose-Con A purification with preparative IEF. Individual isozymes are purified by a factor of ~14 × (there are 14 bands) higher.

Antiesterase immune sera. NSE in 0.1% CTAB extracts of leukapheresed monocytic leukemia cells from patient 1 was purified by Con A-Sepharose 4B affinity chromatography (used for initial inoculation) and preparative IEF (used for boosting). These CTAB extracts from patient 1 contained NSE isozymes in the range of 5.8 to 6.3 pH, but did not contain the pI > 6.3 forms found in other extracts.

New Zealand white rabbits were purchased from Sprague-Dawley. Following inoculation and boosting, the animal was bled every other week. After 4 hours was allowed for clot formation, the serum was removed, heated at 56°C for 30 minutes, and frozen at −70°C.

Immunodiffusion assays of the sera were performed overnight at room temperature in 2% agarose buffered with .05 mol/L of Tris pH 7.2. Unprecipitated protein was dissolved out, and the gels were placed in NSE substrate solution to reveal immunoprecipitation bands containing NSE.

Immunofluorescence of cells was accomplished by testing dilutions of sera. Cells on slides were fixed at 4°C with acetone-methanol (1:1) for 60 seconds and washed in PBS. They exhibited only very weak autofluorescence. Diluted rabbit antisera was added for 1 hour, washed off, and followed by a dilution (1:40) of goat anti-rabbit immunoglobulin (heavy and light chains) (Cappel, Malvern, PA).

Mol wt estimation. Sodium dodecyl sulfate (SDS) polyacrylamide "disc" gels were prepared according to the Laemmli method. The stacking gel was made to 4% acrylamide, and the spacer gel was 7.5%. Samples were preincubated overnight with 0.1% SDS in pH 6.8 Tris-glycine. The gels were run until the bromophenol blue marker left the gel (~20 hours at 20 mA constant current). Protein standards included bovine serum albumin (BSA) (66,000), β-galactosidase (115,000), immunoglobulin G (IgG) (150,000 to 180,000) and thyroglobulin (690,000).

Gel filtration determination of mol wt was performed with G-200 Sephadex and Sephacryl S-300. PBS was the eluant. The ratio of the elution volume and the void volume were linearly correlated with the logarithm of the protein standards mol wt. Protein standards included BSA (66,000), yeast alcohol dehydrogenase (150,000), and β-amylase from potato (200,000).

RESULTS

IEF. IEF separated the isozymes from the other more acidic cellular protein. Analytical IEF revealed that the region exhibiting the NSE staining contained ~1% to 2% of

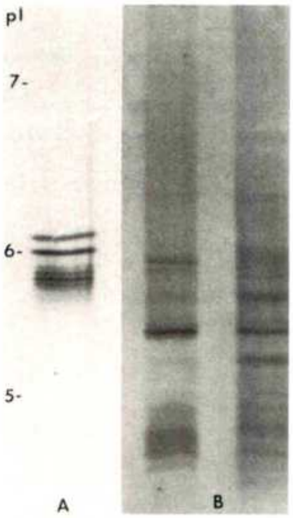

Fig 3. Isoelectric focusing (IEF) of CTAB extract from patient 1 comparing nonspecific esterase (NSE) (A) and Coomassie (B) staining. The left lane in (B) is the same CTAB extract. The right lane is a second CTAB extraction of the same cells.

the total Coomassie staining (Fig 3). Different sets of NSE bands (Fig 4) were obtained from live cells briefly extracted with different detergents or from cells first subjected to freezing.

Brief extraction with CTAB of live cells from patient 1 (2 minutes) yielded two IEF "doublet" bands with esterase activity at pI 6.0 through 6.1 and 6.2 through 6.3 (Fig 4). These cells were mostly intact as judged by electron microscopy and the low lactate dehydrogenase (LDH) content of the extract. These apparent "doublets" were accompanied at pH 5.7 through 5.9 by four bands of weak to strong activity when exposure to detergent was >10 minutes (Fig 4B and C). The pI 6.2 through 6.3 doublet was absent in cells extracted with Triton X-100 (Fig 4C). Longer CTAB extracts (20 to 30 minutes) of live cells, brief CTAB extracts of thawed leukemic cells (frozen in bulk) from patient 1 or cells briefly extracted (2 minutes) by 1% Triton X-100 also displayed a weakly reactive group of six pI > 6.3 doublet bands (Fig 4D) as well as greater LDH activity. These were located at pH 6.60, 6.75, 6.90, 7.05, 7.20, and 7.45 ± .05. Cells reextracted with CTAB yielded only the pI > 6.3 isozymes, but they did so with greater activity (Fig 4E). Leukapheresed cells of patient 2 contained more 0.1% CTAB-soluble pI > 6.3 isozymes than did the cells from patient 1 (Fig 4F). One short extraction (2 to 5 minutes) was sufficient to solubilize nearly all the NSE activity. In IEF gels stained with Coomassie R-250, the pI > 6.3 isozyme bands exhibited a degree of protein concentration similar to that exhibited by the more acidic bands. In contrast to these results with the leukemic cells, extraction of normal monocytes (Fig 4G) yields only the pI 5.7 through 6.3 bands after either 0.1% CTAB or 1% Triton X-100.

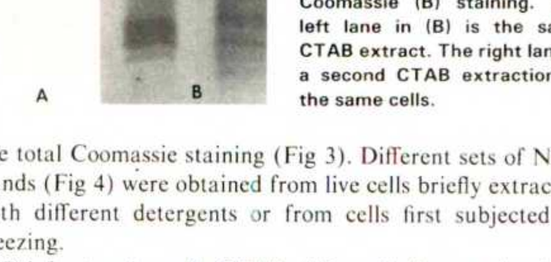

Fig 4. Comparison of representative nonspecific esterase (NSE) staining in IEF gels of: (A) Short extraction (2 to 5 minutes) with CTAB, patient 1; (B and C) longer extractions (10 minutes) with CTAB and short extractions (3 to 5 minutes) with Triton X-100, patient 1; (D) longest extractions (20 to 30 minutes) with CTAB, all extracts from frozen cells, and longer extraction (10 minutes) with Triton X-100, patient 1; (E) cells extracted with CTAB a second time, patient 1; (F) short extractions (2 to 5 minutes) by CTAB or Triton X-100, patient 2; (G) short extraction (2 to 5 minutes) by CTAB of normal monocytes. Dotted lines indicate weaker activity.

Preparative IEF enabled separation of the leukemic isozymes from patient 2 into 2 acid pH regions, 5.7 through 6.0 and 6.0 through 6.3, each containing 4 bands, and 6 neutral regions corresponding to the 6 neutral isozyme doublets. After elution, these preparations were used to test substrate and antisera specificities (see below).

That all the isozymes were glycosylated to some degree is evident from the ability of Sepharose-Con A to bind completely all esterase isozymes from CTAB or Triton X-100 extracts. No difference in binding was exhibited by the different isozymes. The binding of all isozymes was completely reversed by mannose or glucose. The bound enzyme retains considerable activity when bound to Sepharose-Con A even after storage for 1 year at 4°C. Other lectin-affinity chromatography preparations, such as agarose-bound peanut agglutinin or *Lotus tetraglonobus* agglutinin, did not bind or inactivate the enzyme. In contrast, serum cholinesterase, which also hydrolyzes α-naphthyl acetate, was not bound by Sepharose-Con A.

Plasmalemmal 5′ nucleotidase, cytoplasmic lactic dehydrogenase, and acid phosphatase (AcP) of lysosomal origin were present in all preparations. The latter two enzymes were up to fivefold more active or abundant in the most extensive extractions as compared with the shortest CTAB extractions. Most additional solubilized protein in extensive extractions was present in a range between pI 4 and 5.5.

Inhibitors. PMSF, a serine active site inhibitor, is an effective inhibitor of solubilized NSE. Activity in the crude extracts was totally inhibited by 50 mmol/L of NaF, 0.1 mmol/L of PMSF, and pH < 4.5. The I_{50} dose of PMSF varied between 10 and 25 μmol/L depending on the batch.

A degree of differentiation emerged between the pI 5.7 through 6.3 and the pI > 6.3 isozymes in the type of inhibition by PMSF. The inhibition of the neutral isozymes by PMSF was reversed by IEF. These results could be explained by low-affinity noncovalent inhibition of the neutral isozymes, reversed as inhibitors were separated from enzyme by the IEF. The inhibition of all bands by NaF was reversed by IEF.

Neostigmine (10 to 15 mmol/L) inhibited isozymes pI 5.7 through 6.3 30% to 50% when tested with α-naphthyl butyrate in the pH range of 5 through 6. No inhibition of the isozymes was observed in the pH range of 7 through 8. By comparison, serum cholinesterase was inhibited by micromolar amounts, as expected.

Various other compounds were tested as inhibitors of α-naphthyl acetate/butyrate hydrolysis. These have included a wide range of natural and artificial protease and phospholipase inhibitors, none of which had significant effect. Sulfhydryl reducing agents such as dithiothreitol, β-mercaptoethanol and *p*-chloromercuribenzoate; Ca^{2+}/Mg^{2+} chelators like EDTA and EGTA; urea up to 8 mol/L; metal ions like Fe^{2+}, Fe^{3+}, Ni^{2+}, and Mn^{2+}; and the oxidase inhibitor sodium azide all had no effect on soluble or cell associated esterase activity. Isozymes of pI 5.7 through 6.3 were sensitive to 50°C, whereas most pI > 6.3 forms were denatured only after heating to 62°C. These results with solubilized enzyme were cytochemically duplicated by similar treatment of leukemic and normal monocytes. Prefixation of the cells with 0.25% glutaraldehyde decreased the sensitivity of the cellular enzymes to heat. Freezing at −20°C completely preserved the extracted esterase. Dialysis to remove the detergent did not affect activity or the pattern of bands in IEF.

Substrate specificities. We measured the reaction kinetics of the isozymes with α-naphthyl acetate and butyrate at pH 6, pH 7, pH 7.5 and pH 8.5. The leukemic isozymes were separated by preparative IEF as described above. The results are presented in Fig 5 and represent the median of three experiments in which relative absorbance was plotted against pH.

In general, all isozymes, like the crude extract, displayed greatest reactivity between pH 7.5 and 9.0, with an optimum at ~pH 8.5. All isozymes were inactive at pH 9.5 with half-maximum reactivity at pH 9.0 (data not shown). Extracts from normal monocytes behaved very much like the isozymes from leukemic cells. Cytochemically demonstrated NSE exhibited a broader pH optimum from pH 6 to pH 9.

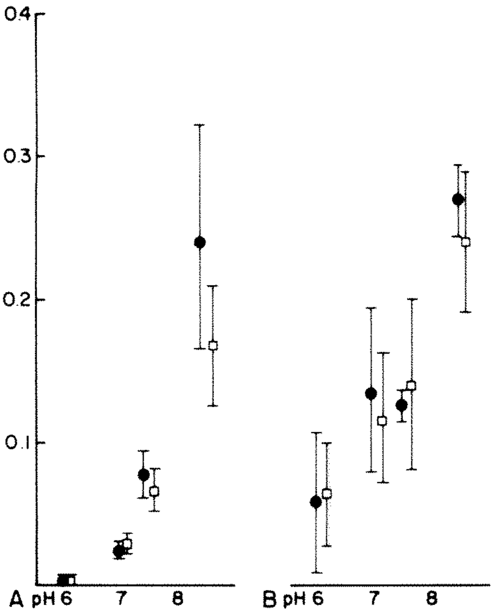

Fig 5. Plot of absorbance of esterase reaction product (measured 500 nm) after 60 seconds (ordinate) as the pH was varied. These graphs represent the average from three experiments of the initial absorbance of each of the separated subgroups of isozymes (described in the Results section) subsequently compiled into the pI 5.7 through 6.3 (□) and the pI > 6.3 (●) isozyme families. The behavior of the enzymes was compared with the α-naphthyl acetate (A) and butyrate (B) substrates. The activity of both families is approximately the same, with a peak around pH 8.5. The activity at pH 9 is approximately one-half that at pH 8.5, whereas at pH 9.5 there is only weak activity (data not shown).

The substrate α-naphthyl butyrate was the most preferred by all isozymes from both normal and leukemic cells, followed in order by α-naphthyl acetate, proprionate, valerate, and caproate. The caproate ester had ~25% the activity of the butyrate as assayed with the crude extract. In IEF gels, the caproate ester was preferentially cleaved by the pI 6.0 isozyme doublet. Crude extract and eluted isozymes of pI 5.7 through 6.0 and 6.0 through 6.3 can also hydrolyze α-naphthyl caprylate, laurate, and myristate, but at ~1% to 2% of the rate of α-naphthyl butyrate, even in a detergent solution.

Mol wt. Gel filtration with Sephadex G-200 or Sephacryl S-300 demonstrated that esterase isozymes exist as units of 50,000 ± 10,000 daltons, which can form aggregates of ~550 kd, presuming that the aggregate is roughly spherical. Aggregates were observed in concentrated solutions of enzyme (after Sepharose-Con A purification) in PBS and when crude detergent extracts were eluted through the columns with PBS. No aggregates were observed when extracts were eluted with detergent solution. IEF showed that the low mol wt fractions from PBS-eluted filtrations were composed of predominantly acid isozyme activities. High mol wt aggregates contained predominantly neutral isozymes. The gel staining was measured only qualitatively.

Discontinuous gel electrophoresis with 0.1% SDS revealed esterase activity only at 150 ± 10 kd, regardless of whether crude detergent extract, Sepharose-Con A purified enzyme, or the 500- to 600-kd G-200 fractions were applied. Because only the activities of pI > 6.3 isozymes survive prolonged exposure to 0.1% SDS, as judged by IEF of crude extract and gel filtration fractions, the 50-kd isozyme monomers (predominantly acid isozymes) could not be visualized with the enzymatic reaction. Because of the remaining contamination with other proteins, even with partially purified preparations, Coomassie staining of protein was not used to localize the NSE.

Antisera. Partially purified extract (composed entirely of pI 5.7 through 6.3 isozymes) from patient 1 was used for immunizing rabbits. The antiserum produced two immunoprecipitin bands containing NSE activity in immunodiffusion assays against the CTAB extract from patient 1. In a similar assay, the extract of the leukemic monocytes from patient 2 exhibited one NSE-positive immunoprecipitin band that possessed an arc of identity with the major band of patient 1 (Fig 6). Immunodiffusion assays using enzyme extracts produced with different detergents (0.1% CTAB, 0.1% CHAPS, 1% Triton X-100) yielded equivalent arcs of identity (Fig 6).

Detergent extracts of normal human Ficoll-Hypaque–purified mononuclear cells or fibronectin-purified adherent cells also possess an arc of identity with the esterase from the leukemic cells (Fig 6). Isozymes separated from preparative polyacrylamide-agarose IEF gels, however, did not react

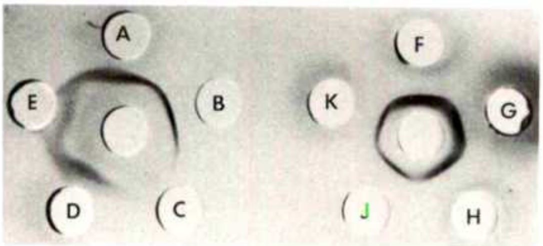

Fig 6. Immunoprecipitation reaction between rabbit anti-pI 5.8 through 6.3 nonspecific esterase (NSE) isozymes (center well) and: CHAPS extract of leukapheresed cells from patient 2 (A), Triton X-100 extract of cells from patient 2 (B), CTAB re-extract of extracted cells from patient 1 (C), CTAB extract of patient 2 (D), CTAB extract of patient 1 (E), CTAB extract of normal monocytes (F and H), Triton X-100 extract of cells from patient 1 (G), CTAB extract of cells from patient 2 (J), and CTAB extract of cells from patient 1 (K). No spurs are present, indicating the similar identity of the different extracts.

equally with rabbit antisera in immunodiffusion plates. Only the pI 5.7 through 6.0 and 6.0 through 6.3 fractions produced NSE-reactive immunoprecipitin bands, exhibiting an arc of identity with one another (Fig 7). The lack of immunoprecipitation reaction with the more neutral bands may be due to the specificity of the rabbit sera immunized with the pI 5.7 through 6.3 isozymes. A different method of analysis, IEF of the isozymes not adsorbed by antisera bound to Sepharose-protein A, revealed the selective loss of the pI 5.7 through 6.3 NSE bands. Thus, there appears to be a difference in the antigenic specificities of pI 5.7 through 6.3 isozymes *v* those with pI > 6.3.

Commercially available antibodies were tested with the extracts. There was no reactivity of NSE with rabbit anti-human serum cholinesterase (Dako, Copenhagen) and goat anti-human C4, C3, or C1 (Sigma). Conversely, immune rabbit serum did not react with serum or erythrocyte cholinesterases or with thyroid esterase. This correlated with the different characteristics of these esterases, including different inhibitor specificities and lack of binding to Sepharose-Con A. The rabbit antiserum did react with a detergent extract of human liver.

Affinity chromatography gels of combined monoblastic leukemia isozymes (derived from Triton X-100 extracts) attached to Sepharose were able to bind the rabbit antisera. Exposure of those gels to pH < 4.5 destroyed esterase activity and, concomitantly, the ability to bind the antisera.

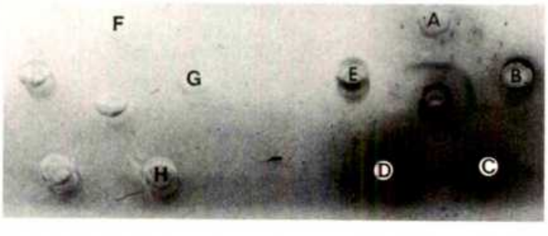

Fig 7. Comparison of immunoprecipitation reaction between rabbit anti-pI 5.8 through 6.3 isozymes (center well) and the pI 5.7 through 5.9 (A), pI 6.0 through 6.3 (B), and six pI > 6.3 isozymes (C through H). The antiserum reacted only with the pI 5.7 through 6.3 isozymes.

Cellular specificity of the antisera was also tested. Dilutions of 1:40 were most effective without too much background. Leukapheresed cells from patient 2 (Fig 8A) were ~90% fluorescent, which matched the proportion of NSE stained cells. Much of the reactivity was probably intracellular. Adherent normal monocytes (Fig 8B) were 50% to 90% positive, depending upon the degree of lymphocyte contamination. Cytocentrifuged nonadherent lymphocytes (Fig 8C) were < 10% positive, correlating with the number of contaminating NaF-sensitive NSE-positive cells, possibly non-adherent monocytes.

DISCUSSION

Leukapheresed mononuclear phagocytes from two patients with acute monocytic leukemia were extracted with detergent to study the solubilized NSE. On the basis of our data, the detergent extractable NSE of the leukemic monoblasts from two patients are separable into two families: the six acid isozymes comprising the pI 5.7 through 6.3 group and the six neutral isozymes with pI 6.60 through 7.45. The common features of both families of enzymes include inhibition by fluoride ion and PMSF, sensitivity to pH < 5, a similar degree of binding to Sepharose-Con A, unit mol wt of 50 ± 10 kd, and similar substrate specificities and pH optima. The distinguishing features of the two groups of isozymes include resistance to elevated temperatures and to 0.1% SDS denaturation, lack of covalent bonding by PMSF to the pI > 6.3 isozymes, and different antigenicity. One further distinguishing feature is our observation that normal monocytes and macrophages (corroborating results of Parawesch and colleagues[8]) possess only the acid isozymes that we find to be antigenically identical to the acid isozymes from leukemic monocytes.

The characteristics of these isozymes are remarkably similar to the liver carboxyl esterases (EC 3.1.1.1). Indeed, a portion of the liver isozymes in most studies was probably contributed by Kupffer cells, which are mononuclear phago-

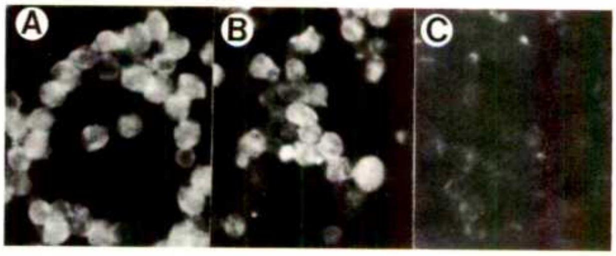

Fig 8. (A) Approximately 90% of the monocytic leukemia cells (90% NSE+, OKMI+) pheresed from patient 2 fluoresce after treatment with 1:40 rabbit anti-NSE serum followed by 1:40 fluoresceinated goat anti-rabbit immunoglobulin. Much of the staining is probably cytoplasmic. Original magnification ×500; current magnification ×375. (B) Adherent normal monocytes were 50% fluorescent in this experiment, which correlated with the number of contaminating lymphocytes present. Original magnification ×500; current magnification ×375. (C) Adherence-purified lymphocytes from normal donors exhibited 5% to 10% bright fluorescence. A number of cells displayed a weak fluorescence. Original magnification ×500; current magnification ×375.

cytes. Like the esterase in our extracts, carboxyl esterases are, by working definition, inhibited by organophosphates but not by the acetyl-cholinesterase inhibitor, eserine, or by sulfhydryl reagents. Relatively concentrated eserine was inhibitory, although that concentration also inhibits neutrophil chloroacetate esterase.[12] Temperature and fluoride sensitivity and pH optimum of beef liver esterases studied by Wynne and co-workers[1] were also similar to our extracts.

Ecobichon[13] demonstrated the presence in human liver of 180- and 65-kd forms of carboxylesterase which existed in the monomeric form below pH 4.5. Horgan and colleagues[14] and Krisch and Krisch,[15] using a variety of biochemical and electron microscopy techniques, showed that pig liver esterase is a 55- to 60-kd unit that can associate as a trimer of 160 kd. Our data indicates a similar unit mol wt in detergent. Without detergent, the pI > 6.3 isozymes polymerized to form a large aggregate of 550 kd, presuming a spherical shape on gel filtration. In 0.1% SDS gels, this ran as an apparent trimer of 150 kd with weak esterase activity. The pI 5.7 through 6.3, 50-kd forms of the enzymes were apparently inactivated by 0.1% SDS.

That all NSE is bound by Con A and is, therefore, glycosylated is evidence of cell surface or Golgi-endoplasmic reticulum lysosome (GERL) localization. In mature monocytes, it has been found only on the cell surface.[2] The neutral to alkaline pH optima of both acid and neutral isozymes is consistent with esterase placement on the cell surface similar to macrophage plasminogen activator and elastase. Because the pH of macrophage phagosomes rises toward pH 8 immediately prior to lysosome fusion,[16] some of the cell surface proteases and NSE isozymes may have a special function during this early step of internalization. Esterase isozymes may also have a function in pinocytosis since we have observed, with electron microscopy, the presence of cytochemical reaction product in pinosomes (unpublished observations, August 1982).

Curiously, the pattern of esterase isoelectric points from leukemic monocytes closely resembles the pattern of platelet (and, presumably, megakaryocyte) esterase described by Radzun and colleagues[10] and Parawesch and co-workers.[8] Because no platelets were present in the leukemic monocyte preparations, there may be some developmental relationship between monocytes and megakaryocyte/platelet bone marrow precursors. One subset of myeloid stem cells, colony-forming units, develops into colonies containing granulocytes, monocytes, and megakaryocytes. Platelets and monocytes do have other cell surface products in common, including complement and fibrin cascade components and fibronectin. Distribution and sharing of esterase isozymes among the various types of leukemias and lymphomas could prove to be a valuable tool in the pursuit of normal and disturbed bone marrow lineage and function.

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Human Plasma Protein Z Antigen: Range in Normal Subjects and Effect of Warfarin Therapy

By Joseph P. Miletich and George J. Broze, Jr

In contrast to the other well-studied vitamin K-dependent proteins that circulate in plasma, protein Z antigen is much more variable. The concentration in plasmas collected in EDTA from 455 normal, healthy donors is normally distributed with a mean of 2.9 μg/mL (46 nmol/L) and a SD of 1.0 μg/mL (95% interval of 32% to 168% of the mean). No significant correlation to age or sex could be detected. In comparison, the concentration of protein C antigen measured with the same type of assay on the same 455 samples has a log normal distribution with a mean of 4.0 μg/mL (65 nmol/L) and a 95% interval of 70% to 138% of the mean. Also in marked contrast to other plasma vitamin K-dependent proteins, the total protein Z antigen level is extremely low in patients on stable warfarin therapy (range 1% to 16% of normal). Moreover, even though >95% of the antigen in normal plasmas adsorbs to barium citrate (a crude reflection of the presence of γ-carboxyglutamic acid (Gla) residues), in the patients taking warfarin almost all of the small amount of the antigen failed to adsorb, suggesting that virtually no protein Z had its full complement of Gla residues. Total protein C antigen in the same 25 patients averaged 53% of normal (34% to 72%) and 54% (average) of the total remaining antigen still adsorbed to barium citrate. The concentration of protein Z antigen in the plasma of a normal individual given a loading dose of warfarin fell at an initial rate of ~20% a day, indicating a plasma half-life ($t^1/_2$) of 2 to 3 days.
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WE PREVIOUSLY described[1] the purification and characterization of a warfarin-sensitive protein found in human plasma. Its apparent mol wt, amino acid composition, amino-terminal sequence, and chromatographic properties are very similar to the bovine protein isolated by Prowse and Esnouf and named protein Z.[2] No physiological function for either the bovine or the human protein has yet been discovered. The complete amino acid sequence of the bovine protein has recently been reported by Højrup and colleagues[3] and reveals extensive homology to the vitamin K-dependent coagulation factors, including 13 Gla residues within the NH_2-terminal 40 residues and one β-hydroxyaspartic acid at position 64. Within the region homologous with the family of serine proteases, however, bovine protein Z, like haptoglobin, does not have an active site serine (changed to alanine) or an active site histidine (changed to threonine). This is consistent with an inability to activate either human or bovine protein Z to a serine protease by limited hydrolysis.[1,3]

To aid in the purification and characterization of human protein Z, we developed six murine monoclonal antibodies. Two of these were used to construct a very sensitive, reproducible, and rapid immunoassay using methods and instrumentation applicable to virtually any immunoassay if appropriate reagents are available. Here we report the wide range of concentrations of protein Z antigen found in the plasmas of normal, healthy subjects as well as the profound decrease in antigen in patients on stable warfarin therapy, both of which are not characteristic of the other well-studied warfarin-sensitive proteins.[4-6] Data on protein C antigen concentration in these same samples, which is much more typical of the other members of this protein family, was measured as part of a separate study and is included for direct comparison. This information is important background in the continued search for the function of protein Z.

MATERIALS AND METHODS

Materials. Affigel-10 was purchased from Bio-Rad Laboratories (Richmond, CA), and cyanogen bromide-activated Sepharose 4B, Sephacryl S-300, and Sephadex G-25 were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Repligen (Cambridge, MA) was the source of recombinant Protein A. Bovine serum albumin (BSA), goat anti-mouse IgG alkaline phosphatase conjugate, Trizma base, fluorescein isothiocyanate (FITC), and latex beads (0.8 μm) were bought from Sigma Chemical (St Louis). Dulbecco's modified Eagle's medium L-glutamine, nonessential amino acids, penicillin/streptomycin, and bovine and horse sera were obtained from KC Biological (Lenexa, KS). Epicon assay plates, Epicon Performance Verification Particles, and Epicon Reference Particles (590/620 nm) were purchased from Pandex Laboratories (Mundelein, IL). All other chemicals were reagent grade or better and came from Sigma or from Fisher Scientific (Pittsburgh).

Plasmas. Plasma samples used to determine the distribution of protein Z antigen in the normal population were collected from participants in health fairs, in 5-mL Vacutainer tubes (Vacutainer Systems, Rutherford, NJ) that contain 48 μL of 15% K_3 EDTA anticoagulant. Cells were removed by centrifugation at 3,000 g for 15 minutes and the plasmas were quick-frozen at −85°C. Serum was also obtained for routine chemistries. Subjects were considered normal if no more than three of the following laboratory tests were further than 2 SD from the normal mean and all results were within 3 SD of the mean: electrolytes (sodium, potassium, chloride, and total CO_2); standard blood chemistry (blood urea nitrogen, creatinine, total bilirubin, uric acid, glucose, total calcium, serum phosphate, total protein, albumin, and cholesterol); enzymes (alkaline phosphatase, γ-glutamyl transpeptidase, aspartate aminotransferase, total lactate dehydrogenase, and creatine kinase); and routine hematology (erythrocyte count, hemoglobin, packed RBC volume, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, RBC distribution width, WBC count, lymphocyte count, monocyte count, granulocyte count, platelet count, and mean platelet volume). Subjects also answered a

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questionnaire; there was no indication of concurrent antibiotic usage, liver disease, anticoagulant therapy, or hemorrhagic or thrombotic disorders in any of the individuals included in this study. Participants were informed that their blood samples would be used in normal range studies; samples and histories were identified only by a coded number and not by name to insure confidentiality.

Plasma samples from blood donors randomly selected during a 3-week interval were generously provided by Dr Laurence Sherman of the St Louis Bi-State Chapter of the American Red Cross. After a unit of blood is collected from a donor, one sample for serum and one for cells and plasma (EDTA anticoagulant) are routinely taken (using Vacutainer tubes and a separate port of the same line) for testing at the Red Cross. The anticoagulated samples that were processed within 24 hours from the time of collection and that were of no further interest to personnel at the Red Cross, from donors with no evidence of infectious disease, were used to recover plasmas as described above. Samples were identified only by a bar-coded number and were not identified in any manner by donor name. Because donors must wait at least 8 weeks between donations, all samples were from different persons. The first day's samples ($n = 429$) were pooled (1.0 mL from each) and aliquotted as a normal control standard; samples from subsequent days were individually analyzed.

Citrated plasma samples from 25 outpatients taking warfarin for various cardiac abnormalities were recovered from blood sent to the clinical hemostasis laboratory for routine monitoring of the prothrombin time. Each patient's record was checked to insure that therapy had been continuous and uneventful for at least 6 months with no medical intervention for other illnesses.

Four different lots of pooled normal plasma (citrate anticoagulant, 20 donors) were obtained from George King Biomedical (Overland Park, KS).

Plasma containing $<1\%$ protein Z was prepared by immunoadsorption using either monoclonal antibody HZ-1 or HZ-7 attached to Affigel-10 at a concentration of ~ 10 mg/mL according to the manufacturer's directions. Five-milliliter (1.1×5 cm) columns of the immunoadsorbant were equilibrated in normal saline buffered with 20 mmol/L of Tris at pH 7.4. Ten milliliters of unbuffered saline followed by 25 mL of pooled normal plasma (George King Biomedical) was passed through the columns at rates of 1 mL an hour for HZ-1 and 5 mL an hour for HZ-7. The saline and the first 5 mL of plasma eluted were discarded; the next 17 mL were pooled. Neither plasma had any detectable protein Z antigen as compared with a buffer control as measured with the standard two-site assay or the barium citrate adsorption assay (using either monoclonal antibody) as described below. In addition, both plasmas were free of protein Z antigen as assessed by Western blot analysis using a polyclonal antibody (raised in a goat) with a sensitivity of 0.1 ng ($<1\%$ of normal plasma given the 5 μL vol tested).

Estimates of affinity constants. Two methods were used. In the first (the free antigen method), antibody was held constant at a concentration known by trial to be close to its K_d. Purified protein Z (in Tris-buffered saline containing 100 μg/mL bovine serum albumin, BSA) was varied from roughly one-fifth K_d to $5 \times K_d$. After incubation for sufficient time to establish equilibrium at the lowest antigen concentration, latex particles coated with goat anti-mouse IgG were added in large excess (enough to adsorb at least 5 times the amount of mouse antibody present in 10 minutes). Monoclonal antibody specific for protein C was used to correct for nonspecific adsorption of protein Z. After removal of the particles by centrifugation at 12,000 g for 1 minute, the concentration of antigen in the supernatant samples was determined using the standard assay described below. Bound antigen was calculated and K_a was estimated from the intercept of a double-reciprocal plot. The second method (sequential saturation) was essentially the same except that incubations were set up (replicates of eight) in Epicon plates and the Screen Machine added the goat anti-mouse particles. Two minutes before collection of the particles, FITC-labeled protein Z was added to each well. With this method, bound fluorescence is proportional to the concentration of free antibody sites. Background fluorescence was determined from wells containing no monoclonal antibody, and the fluorescence corresponding to the total concentration of antibody sites was determined from wells containing no unlabeled protein Z. Calculations were performed on the IBM PC using software supplied by Pandex Laboratories (Mundelein, IL), but essentially involve Scatchard analysis of the bound and free concentrations, assuming that the labeled probe does not significantly alter the equilibrium protein Z and the antibody during the 2-minute pulse.

Monoclonal antibodies. Standard methods for immunizing Balb/C mice, fusing splenic WBCs to Sp 2/0-Ag14 mouse myeloma cells with polyethylene glycol (PEG), and isolating hybridomas with selective growth media were followed. Supernatant samples were tested with an enzyme-linked immunosorbent assay (ELISA) using goat anti-mouse IgG conjugated to alkaline phosphatase and wells coated with protein Z, protein C, protein S, factor IX, or prothrombin. Cell lines positive and specific for protein Z were subcloned on soft agar; positive subclones were subjected to at least one freeze/thaw cycle. Surviving hybridomas that still secreted specific antibody to protein Z after several weeks in culture were selected for further characterization.

Proteins. Protein Z and thrombin were isolated as previously described.[1,7] Thrombin-treated protein Z was prepared by incubating 1.0 mg/mL of protein Z with 70 μg/mL of α-thrombin in 0.1 mol/L of NaCl, 20 mmol/L of Tris, pH 8.0, for 3 hours at 37°C. The thrombin-cleaved protein Z (Z_t) was separated from both thrombin and uncleaved protein Z by chromatography using a Pharmacia fast protein liquid chromatography (FPLC) Mono Q column equilibrated in 0.1 mol/L of NaCl, 20 mmol of MES, pH 6.5, and developed with a linear NaCl gradient.

Murine monoclonal antibodies were purified from ascites using protein A-Sepharose 4B prepared from recombinant protein A and cyanogen bromide activated Sepharose 4B, according to the manufacturer's instructions (2.5 mg protein A per mL of packed gel). IgG was eluted with 0.1 mol/L of sodium citrate, pH 3.5, dialyzed against 0.1 mol/L of NaCl, 10 mmol/L of HEPES, pH 7.5, and stored at −85°C.

Proteins to be fluorescein-labeled were dialyzed against 0.1 mol/L of sodium phosphate, pH 7.4, and adjusted to a concentration of 1 mg/mL. FITC was dissolved in freshly prepared 0.5 mol/L of sodium carbonate, pH 9.5, at a concentration of 0.8 mg/mL and added to the antibody at a ratio of 1:4 (vol/vol). After 2 hours of gentle mixing at room temperature, the labeled protein was separated from unbound fluorescein by chromatography through at least 5 vol of Sephadex G-25, equilibrated in 0.15 mol/L of NaCl, 20 mmol/L of Tris, pH 7.4. The molar extinction coefficient for FITC was taken to be 80,000 at 495 nm; the concentration of protein was calculated from the optical density at 280 nm after subtraction of 0.35 times the optical density at 495 nm to correct for the fluorescein, using an extinction coefficient of 14.5 for antibodies and 12.0 for protein Z. Between 5 and 12 mol of fluorescein were incorporated per mol of protein by this method.

Instrumentation. Plasma samples were handled with a Tecan model 505 Robotic Sample Processor (Tecan US, Chapel Hill, NC). The precision of pipetting 5.0 μL was $>1\%$ over a wide range of plasma viscosities; the drawn Teflon pipetting tip was washed inside and out with 2.0 mL of saline between samples, and no carryover could be detected. A 96-well Epicon assay plate can be loaded with standards, samples, and controls in <15 minutes; care was taken to guard against sample evaporation even within this short time.

Sample analysis was performed with a Screen Machine (Pandex).[8] The well-to-well coefficient of variation for all steps of particle and reagent addition, separation, washing, and quantitation as determined by using latex particles impregnated with fluorescent dyes is 4% to 8% for single-channel fluorescence and <2% when the ratio of fluorescence in two channels is calculated using reference particles. Raw data were transmitted to an IBM PC for storage and processing, using software supplied by Pandex. Linear regression coefficients were calculated using the least-squares method.

Assays. Standards for assays of protein Z antigen in plasma were prepared by combining purified protein Z (1.12 mg/mL) and the pooled plasma that had been depleted by immunoadsorption in appropriate volumes to give final concentrations of 0, 1, 2, 4, and 6 μg protein Z/mL; 100-μL aliquots were stored at −85°C and were discarded after a single day's use.

For the standard assay of total protein Z, plain latex particles (0.5% wt/vol) mixed with reference particles (0.01% wt/vol) were coated with monoclonal antibody HZ-7 (100 μg/mL) in 0.1 mol/L of sodium phosphate, pH 5.5, for 2 hours with gentle agitation at room temperature, and then stored for up to 2 weeks at 4°C. Just prior to use, the required volume of coated particles was collected by centrifugation for 2 minutes at 12,000 g; the supernatant was replaced with twice the volume of TBSA. The coated particles were thoroughly dispersed in an ultrasonic bath. Five microliters of plasma sample diluted with 15 μL of TBSA were placed in the Epicon assay wells. The coated particles (20 μL) were added to each well by the Screen Machine, followed 10 minutes later by 20 μL of fluorescein-labeled monoclonal antibody HZ-1 at a concentration of 10 μg/mL in TBSA. After an *additional* 20-minute incubation, the particles were collected on the filters, washed twice with 50 μL of Tris-buffered saline and analyzed for bound fluorescence. All reactions were at room temperature. Separate experiments demonstrated that the reaction of both antibodies with protein Z was >90% complete in 10 minutes under the assay conditions. Further increase in signal was negligible at longer incubation times. The actual timing of addition of the reagents partially reflects a minimization of total assay time for the instrument, which can handle up to ten plates in the same batch.

For the assay of barium-adsorbable antigen, 20 μL of the appropriate fluorescein-labeled antibody (or antibodies) at a concentration of 10 μg/mL in buffer containing 75 mmol/L of sodium citrate, 40 mmol/L of NaCl, and 100 μg/mL BSA, pH 7.4, was added to samples diluted as above, followed in 20 minutes by 20 μL of 150 mmol/L of $BaCl_2$. After 5 additional minutes, the barium citrate particles were collected, washed once with 50 μL of buffer (50 mmol/L of $BaCl_2$, 100 mmol/L of NaCl, 20 mmol/L of Tris at pH 7.4) and analyzed for bound fluorescence. These conditions were found to "capture" >95% of the vitamin K-dependent antigens reproducibly whether the plasma was initially anticoagulated with citrate or EDTA. Reference particles could not be included as an internal standard, however, since they interfered in an unknown manner with the formation of barium citrate particles that were retainable by the filters.

RESULTS

Characterization of monoclonal antibodies. Six hybridomas from three separate fusions survived storage in liquid nitrogen and continued to secrete specific antibody in long-term culture. Each antibody was evaluated for cross-reactivity with the other antibodies, for affinity toward protein Z and apparent kinetics, and for its ability to retain reactivity when labeled with FITC or when bound to latex particles. One antibody (HZ-1) was clearly superior as an FITC-labeled probe for detecting bound protein Z; HZ-7 had the best characteristics for a "capture" antibody and was not cross-reactive with HZ-1. Both antibodies are IgG_1-κ. The estimates of K_a were 4.6×10^7 and 5.7×10^7 mol/L^{-1}, and 5.2×10^8 and 2.7×10^8 mol/L^{-1} for HZ-1 and HZ-7, respectively, by the free antigen and the sequential saturation methods (described in the Materials and Methods section). Both antibodies are specific for protein Z by Western blot analysis of plasma (data not shown) and detect only protein Z in the protein fraction adsorbed from plasma with barium citrate (see below). By Western blot, these antibodies also recognize Z_t, which is missing the NH_2-terminal Gla-domain (described in Discussion section) and migrates slightly faster in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system.

Assays for human protein Z. The two antibodies were used as described in the Materials and Methods section in a two-site particle concentration immunoassay. The data shown in Fig 1 demonstrate that (a) fluorescence is directly and linearly proportional to the concentration of purified protein Z whether it is diluted in buffer or in the plasma immunodepleted of protein Z, and (b) that the protein Z in pooled normal plasma behaves in exactly the same fashion as purified protein Z, regardless of whether it is diluted in immunodepleted plasma or in buffer. No difference was detected between plasmas immunodepleted with either antibody. Protein Z missing the NH_2-terminal Gla-domain (Z_t) reacted normally in the assay, demonstrating that neither antibody is sensitive to changes in that region of the protein. The overall reproducibility of the assay was evaluated (using reference particles to correct for minor changes in well geometries) by assaying the normal pool (EDTA) in duplicate ten separate times on different days using the same reagents; the mean was 2.77 μg/mL with a SD of 0.04 μg/mL (calculated coefficient of variation of 1.3%). Even

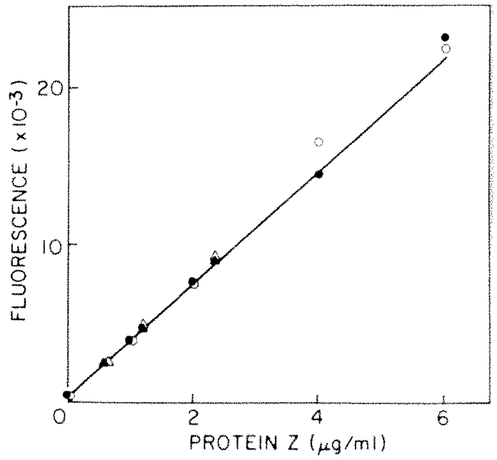

Fig 1. Standard curve for protein Z antigen in buffer v depleted plasma. Different amounts of purified protein Z were added to either buffer (○) or plasma immunodepleted of Protein Z (●), and the fluorescence was determined in the latex particle immunoassay (described in the Materials and Methods section). Normal pooled plasma (citrated) samples were assayed at the same time undiluted and diluted 1:1 and 1:3 with buffer (△) or with depleted plasma ▲. Each point is the average of duplicate determinations.

when different preparations of reagents and standards were used, the coefficient of variation of the assay did not exceed 4%. The signal was linear with increasing protein Z up to a concentration of 10 μg/mL when samples were processed as described. The detection limit for the assay as described is 10 ng/mL (signal ≥ mean background + 1 SD), although this can be extended by simply using larger sample volumes.

More than 95% of the protein Z antigen in normal plasma can be adsorbed to barium citrate. Barium citrate particles are retained by 0.2-μm filters. Therefore, it seemed reasonable to use barium citrate to "capture" protein Z (as well as all the other vitamin K-dependent proteins) and quantitate it using either of the specific FITC-labeled monoclonal antibodies. Both antibodies gave very similar, linear standard curves and each detected the same amount of protein Z antigen (data not shown). The standards are made up in immunodepleted plasma that still had normal concentrations of the other barium-adsorbable proteins, thus attesting to the specificity of each antibody. When both FITC-antibodies were included, the fluorescence signal was additive, proving again that the antibodies recognize distinct epitopes and do not interfere with one another's binding. Furthermore, neither antibody prevents protein Z from binding to barium citrate or vice versa. These assays are not as reproducible (coefficient of variation ~10%) as the two-site latex particle assay described above due to the added variability of barium adsorption and filtration and the lack of an internal reference standard, but they serve to reinforce the fact that each of the antibodies used detects the same antigen in normal plasma. When Z_t was added to buffer or to immunodepleted plasma, it failed to adsorb to the barium citrate (data not shown), as might be expected since it is missing the Gla-domain. Thus, although both antibodies bind to Z_t, neither could detect it in the barium adsorption assay since the protein and antibodies react but pass through the filter without binding to the barium citrate.

Distribution of plasma protein Z antigen in the normal population. The plasma concentration of protein Z antigen was determined in 455 normal, healthy adults (aged 19 to 80 years). Each sample was assayed in duplicate all on the same day to reduce assay variability to the absolute minimum. The mean level was 2.87 μg/mL with a SD of 1.00 μg/mL and a range of 0.63 to 5.67 μg/mL. Protein C antigen was determined in the same samples (using virtually identical methods and conditions) using two monoclonal antibodies specific for protein C (HC-2 and HC-4). In Fig 2, the data are plotted as frequency histograms with concentrations expressed as percentages of the means. The lines designate the predicted population frequency assuming a normal distribution for protein Z and a log normal distribution for protein C. Variability between individual plasma levels is at least two times greater for protein Z than for protein C, the latter being more typical of other known vitamin K-dependent plasma proteins. Furthermore, there was virtually no correlation between the concentrations of protein Z and protein C in these samples, when each was expressed as a percentage of the population mean (coefficient of correlation [COC] = 0.13). There was also no correlation of protein Z antigen concentration to age or gender (data not shown). Four different lots of citrated pooled normal plasma assayed in quadruplicate had mean antigen levels of 2.36 (GK 411C), 2.48 (GK 920C), 2.55 (GK 317), and 2.30 (GK 928) μg/mL; the overall mean, 2.42 μg/mL, is 84% of the mean for the 455 samples collected in EDTA anticoagulant, which is very close to the value of 83% calculated from plasma dilution by the citrate anticoagulant assuming a mean packed RBC volume of 44%. This also agrees well with the estimate of 2.2 μg/mL in citrated samples we previously reported using a rabbit antisera and electroimmunodiffusion.[1]

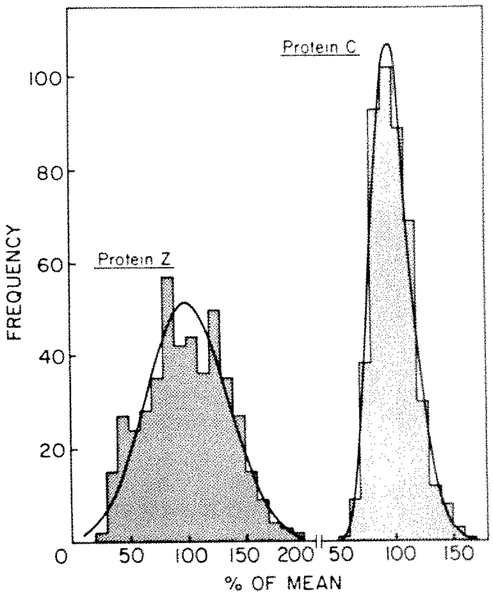

Fig 2. Distribution of protein Z and protein C antigen in normal plasmas. The concentration of protein Z and protein C in the plasmas (EDTA) of 455 normal subjects was determined using latex particle immunoassays. Data are plotted as frequency histograms using intervals of 10% of the mean for all samples. Lines indicate predicted distributions. Best fits were obtained using untransformed data for protein Z and log-transformed data for protein C.

These normal range studies did not include infants, children, or adolescents, but 18 samples obtained from neonates (mean age 11 days, range 3 to 28 days) were checked. These newborns were hospitalized for various clinical conditions and cannot necessarily be taken as representative of normal healthy neonates. Nonetheless, protein Z antigen averaged 57% ± 23% (SD) of the normal adult level with a range of 29% to 107%. This was significantly higher and more variable than protein C antigen, which averaged 28% ± 8% (SD) of the normal adult level with a range of 21% to 48%. There was again a very limited correlation (COC = 0.29) between the two antigen concentrations.

Decrease in plasma protein Z antigen with warfarin treatment. Citrated plasma samples from 25 outpatients receiving stable warfarin therapy without incident for at least 6 months were analyzed for protein Z and protein C antigen (Table 1). Although the total amount of protein C antigen was decreased by only ~50%, total protein Z antigen was reduced nearly 13-fold. Making the samples 5 mmol/L in EDTA made no significant difference in the protein Z

Table 1. Protein Z and Protein C Antigen in 25 Patients Taking Warfarin

	Protein Z (% of Normal)		Protein C (% of Normal)	
	Total	Barium Adsorbable	Total	Barium Adsorbable
Mean ± SD	8 ± 4	1 ± 2	53 ± 8	28 ± 6
Range	1–16	0–7	34–74	18–44

Normal pooled plasma (citrate) was analyzed in quadruplicate in each assay. The average values were 2.31 μg/mL total and 2.27 μg/mL barium adsorbable for protein Z and 2.96 μg/mL total and 2.93 μg/mL barium adsorbable for protein C.

assay. When protein Z antigen was "captured" by adsorption to barium citrate and quantitated with either monoclonal antibody (HZ-1 shown), the mean concentration was only 0.02 μg/mL (1% of normal), indicating that most of the little remaining antigen is also deficient in Gla residues; 15 of the 25 samples had no detectable protein Z antigen in this assay. Similar assays using either of the monoclonal antibodies specific for protein C (HC-2 shown) demonstrated that roughly half the total amount of this antigen still had sufficient Gla residues to bind to barium citrate. Because both HZ-1 and HZ-7 react equally well (but at different epitopes) with Z_t that has the Gla-domain completely removed, it is extremely unlikely that the binding of both is somehow adversely affected by deficiency of γ-carboxylation of Gla residues in that region. These data indicate that protein Z antigen in plasma is markedly more sensitive to chronic warfarin therapy than is protein C or the other vitamin K-dependent plasma proteins.[6-8]

Using the electroimmunodiffusion assay we previously reported that protein Z antigen becomes undetectable after 3 to 5 days of warfarin therapy, but we could not then absolutely distinguish a decrease in total antigen from a possible loss of reactivity with the antisera due to decreased carboxylation (although we recognized that a complete loss of reactivity would be unusual for a polyclonal antibody). The disappearance of antigen was investigated more closely with the assays described here, since neither of the monoclonal antibodies requires the presence of Gla residues for reactivity. Twenty months prior to the current experiments, one of us (J.P.M.) took warfarin during studies of human factor VII.[7] Samples had been saved at −85°C and were assayed for protein Z antigen along with a freshly drawn, never-frozen sample (Fig 3). Several features are apparent. First, the plasma concentration of protein Z antigen in a healthy individual shows no significant variation throughout the day or across at least 20 months. Second, protein Z antigen is stable when frozen at −85°C for at least 20 months. Third, when plasma protein Z antigen falls in response to loading dosages of warfarin, the apparent plasma half-life (t½) is 2 to 3 days; the increase in the plasma level following administration of vitamin K is similar. Fourth, in this setting of short-term vitamin K antagonism, the concentration of antigen adsorbable to barium citrate parallels total antigen, showing that very little "Gla-less" protein Z appears in plasma. Therefore, the total contribution of newly-synthesized protein Z antigen in the plasma is negligible, which means that the true biological t½ of total antigen is in fact several days. Whether this is the true clearance rate from plasma or there exists a significant extravascular pool of protein Z antigen in equilibrium with plasma cannot be determined from these experiments. It should be noted that the protein Z antigen in this experiment never reached the same extremely low levels observed in patients on chronic, lower doses of warfarin because vitamin K was administered after 2 days when the factor VII level was <1%. Presumably, the protein Z antigen would have continued to decrease otherwise.

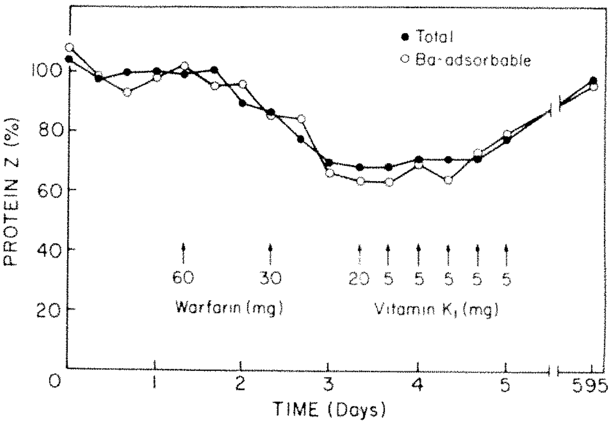

Fig 3. Effect of short-term warfarin administration on protein Z concentration. Protein Z antigen in the plasma (citrated) of a normal subject was determined with both the latex (●) and barium citrate (○) particle immunoassays. Warfarin and vitamin K_1 were administered orally at the times shown. The value of 100% was calculated as the average of the first five samples.

Verification of wide range of protein Z antigen in normal subjects. Twenty-five hundred blood samples (EDTA anticoagulant) collected during a 3-week interval were generously supplied by the American Red Cross (described in the Materials and Methods section). The distribution of antigen concentrations was the same as described above, ie, approximately normal with nothing to suggest the presence of subpopulations. Nevertheless, 18 of the plasmas with very low and 18 with very high antigen concentrations were analyzed in more detail, as shown in Table 2. The measured level of protein Z was the same in every case regardless of whether the two monoclonal antibodies were used separately or together. Therefore, the wide range of concentrations found in normal subjects cannot be an assay artifact based on the presence of a polymorphism in the population unless it affects two independent epitopes to exactly the same extent. Within the limits of the assays, the protein Z antigen in every case was almost completely adsorbable to barium citrate as well, making it unlikely that protein Z can be synthesized independent of vitamin K in some individuals and therefore cause the wide range of values. Total protein C antigen measured in each group was entirely normal and showed the expected distribution, again verifying that there was no correlation between protein Z and protein C concentrations.

The possibility that some other substance(s) in plasma somehow affected the amount of antigen detectable by the antibodies (eg, by binding to protein Z) was also considered. Mixtures of two plasmas (with 31% and 179% antigen,

Table 2. Protein Z and Protein C Antigen in Plasmas With Extremely High or Low Concentrations of Protein Z

	Protein Z (% of Normal)			Protein C (% of Normal)
		Barium Adsorbable		
	Total	HZ-1	HZ-7	Total
High (n = 18)				
Mean ± SD	185 ± 21	187 ± 27	186 ± 26	104 ± 16
Range	152-230	140-222	149-230	79-143
Low (n = 18)				
Mean ± SD	27 ± 3	24 ± 4	19 ± 5	97 ± 18
Range	20-34	15-31	10-30	65-145

Plasmas (EDTA) with either high or low concentrations of protein Z were selected from 2,500 samples taken from blood donors. Duplicate determinations were averaged and expressed as a percentage of the mean for the 455 control samples used for the study of normal range.

respectively) were made, resulting in molar ratios from 0 to 1 in increments of 0.1. After a 2-hour incubation at 37°C, the mixtures were analyzed. The response was linear ($r^2 = .993$), indicating that if an interfering substance exists in one of the plasmas it is either incapable of interacting with protein Z from the other plasma or it is present in some limiting amount.

To address the latter possibility, the pooled control plasma (EDTA) was chromatographed on a column of Sephacryl S-300 equilibrated in the assay buffer. Only a single peak of antigen (eluting after IgG but slightly before protein C and albumin) was detected in the fractions. Thus, the pooled plasma did not contain a significant concentration of a substance of appreciable mol wt that could bind to protein Z in the assay buffer. Therefore, the wide range of protein Z antigen concentration observed in normal plasmas is not likely to be an artifact based on the presence of variable quantities of interfering substance(s) in some plasmas.

Finally, the possibility that protein Z was altered in some plasmas by the procedures for obtaining plasma was considered. Because protein Z is specifically cleaved by thrombin, which could conceivably be present at very low concentrations at the time of venipuncture, we tested Z_t in the standard assay as mentioned previously; it was indistinguishable from native protein Z. Neither antibody detected any trace of Z_t in the barium citrate adsorption assays, however, since Z_t does not bind to barium citrate. Therefore, not only would Z_t be detected anyway in the standard assay if present in some samples, but it would also cause a discrepancy between the two types of assays, which was never observed with normal plasmas. In other experiments, the serum and plasma levels of protein Z were not different, storage of blood samples for at least 5 days (at room temperature or at 4°C) before separation of the plasma caused no change in concentration, and collecting blood into an inhibitory "cocktail" containing aprotonin, diisopropylfluorophosphate, hirudin, soybean trypsin inhibitor, and benzamidine made no difference.

DISCUSSION

The function of protein Z remains unknown. It seems unlikely that human protein Z or any portion of it has enzymatic activity. It may be a cofactor similar to protein S, which also lacks enzymatic activity. It may have nothing to do with hemostasis; the evidence linking protein Z to blood coagulation at this time is its structural similarity to six other vitamin K-dependent plasma proteins (factors II, VII, IX, and X, and proteins C and S).

The concentration of protein Z antigen found in 95% of adult plasmas varies from 0.9 to 4.8 µg/mL (32% to 168% of the mean), with an actual observed range of 0.6 to 5.7 µg/mL. This distribution is considerably wider than has been found for the other vitamin K-dependent plasma proteins. No correlation of concentration to age or sex could be detected, although any minor dependence could be easily obscured by the nearly tenfold range in normals. Protein Z is also unique in its sensitivity to chronic warfarin therapy, which reduces the concentration of total plasma antigen to 8% of normal. Furthermore, even though nearly all of the antigen in normal plasmas will adsorb to barium citrate, suggesting that at least some of the Gla residues are present in every molecule, 60% of samples from patients taking warfarin chronically have no detectable adsorbable antigen. Whatever the function of protein Z may be, any given individual's plasma concentration clearly need not be maintained very near the population mean for any essential physiological activity. Whether this marked drop in antigen level in patients taking warfarin may be of clinical interest in some special settings (eg, monitoring compliance or refractoriness to warfarin therapy) has not yet been evaluated.

The possibility that protein Z is not a plasma protein in the sense that plasma is its site of action must be considered. If non-liver tissue (eg, bone, endothelium, etc) is the site of synthesis *and* of function for protein Z, and if plasma levels only reflect an average clearance, this could conceivably explain the wide range in normals, the greater sensitivity to warfarin, and the inability to find a function using plasma-based in vitro assay systems. A limited analogy for this concept might be the iron system. Ferritin can be measured in plasma even though it "functions" principally extravascularly, and it has a tenfold range of normal (25 to 240 and 12 to 130 ng/mL for adult men and women, respectively, in our laboratory). Transferrin, on the other hand, has a much tighter normal range (204 to 360 mg/dL) and acts principally intravascularly. When the role of protein Z is discovered, it will be essential to bear in mind the wide variation in plasma concentration in normal subjects before attempting to correlate any clinical problems with protein Z "deficiency" based on measurements in plasma.

The observation that thrombin-cleaved protein Z does not bind to barium citrate is interesting because cleavage of protein S by thrombin causes a similar change in that protein.[9] The cleavage sites are not homologous, however. Dahlbäck and colleagues[10] showed that bovine protein S has a region between residues 45 and 75 that is not homologous to the other vitamin K-sensitive proteins in plasma. Thrombin hydrolyzes two bonds (at arginines 52 and 70) releasing an 18 amino acid peptide; the NH_2 terminus containing the Gla residues remains linked by a disulfide bond (involving cysteine 47) to the rest of the molecule. Human Z_t on the other hand has only a single new NH_2-terminus that is identical in 10 of 14 residues with the sequence of bovine protein Z beginning with residue 42 (G.J. Broze and J.P. Miletich, unpublished observations, May 1986). If a function for protein Z is discovered, cleavage by thrombin or other proteases may be a means of regulating interactions dependent on the presence of the Gla residues.

The two-site monoclonal antibody particle concentration fluorescence immunoassay described in this study has favorable characteristics when compared with other antigen assays. Sample requirement is low (5 μL), sensitivity is high (limit of detection <1% of normal), and reproducibility is great (coefficient of variation <2%). Data handling is simple since signal is directly and linearly proportional to concentration over the entire range, with very low background. Sample throughput is substantial; one person using the equipment described can assay ~80 samples an hour in duplicate. There are no toxic or radioactive substances, and the reagents have proved stable for 6 months, with no indication of deterioration as yet. Similar assays developed for factor VII and factor IX share these characteristics, indicating that many antigens of clinical interest can be measured rapidly and accurately in large numbers using these techniques.

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Differential Binding of Erythroid and Myeloid Progenitors to Fibroblasts and Fibronectin

By Schickwann Tsai, Vikram Patel, Elisabeth Beaumont, Harvey F. Lodish, David G. Nathan, and Colin A. Sieff

Using a novel coverslip-transfer culture technique, we recently demonstrated that primitive erythroid burst-forming units (BFU-E) can migrate, proliferate, and differentiate in intimate association with stromal fibroblastoid cells in the presence of serum proteins and erythropoietin. No other exogenous hemopoietic growth factors are required. Most of the colonies that develop in this system are very large erythroid bursts, and very few granulocyte-macrophage (GM) colonies are observed. In this report, we present data indicating that the predominance of erythroid burst colonies in this culture system is due to preferential binding of primitive erythroid progenitors to the stromal fibroblastoid cells and not to differential stimulation of these erythroid progenitors by these cells. We next show that the binding of BFU-E to stromal cells is blocked by anti-fibronectin antibodies. Finally, we demonstrate the preferential binding of BFU-E to fibronectin by using glass coverslips or Petri dishes coated with purified human plasma fibronectin. The binding is blocked by a monoclonal antibody specific for the cell-binding domain of fibronectin. We conclude that: (a) primitive erythroid progenitors bind strongly whereas G and/or M progenitors (CFU-G/M) bind only weakly to fibronectin; (b) primitive erythroid progenitors bind to the cell-binding domain on the fibronectin molecule; and (c) erythroid progenitors and precursors remain bound to fibronectin throughout differentiation.
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THE PROLIFERATION and differentiation of hemopoietic progenitors in short-term colony assays is dependent on exogenous hemopoietic growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and erythropoietin.[1,2] For long-term liquid culture of bone marrow, an adherent stromal layer consisting mostly of fibroblastoid cells, macrophages, and the extracellular matrix is essential for sustained hemopoiesis over several months.[3,4] The exact functional roles of the stromal cells and matrix are poorly understood, however, due to the complexity of the hemopoietic microenvironment and the lack of culture systems suitable for detailed analysis.

Uninduced murine erythroleukemia (MEL) cells grown in suspension culture attach tightly and specifically to fibronectin, and dimethyl sulphoxide-induced erythroid differentiation of these cells is accompanied by parallel loss of cell adhesion to fibronectin and fibronectin receptors.[5,6] MEL cells induced in suspension culture rarely undergo enucleation but cells induced on fibronectin coated dishes enucleate to become reticulocytes, suggesting that interaction with fibronectin is essential for completion of the erythroid differentiation process in vitro.[7] Fibronectin is a glycoprotein component of extracellular matrices of many tissues, and it promotes attachment of many types of cells in vitro.[8] Fibronectin has also been implicated in cell migration during embryogenesis[8,9] and in myogenesis.[10] Fibronectin is a major component of the extracellular matrix in bone marrow.[11] Whether erythroid progenitors in bone marrow do indeed interact with fibronectin remains to be determined.

Recently, we introduced a new "coverslip-transfer culture" system, designed to study the functional roles of stromal cells in hemopoiesis.[12] In this system, human bone marrow cells depleted of monocytes, macrophages, and T cells are allowed to adhere to monolayers of a homogeneous fibroblastoid human stromal cell strain (ST-1) grown on coverslips. The coverslips are then rinsed to remove nonadherent bone marrow cells, transferred to a new culture dish, and overlaid with methylcellulose culture medium containing fetal calf serum (FCS) and erythropoietin. No other exogenous hemopoietic growth factors are added. In this culture system, primitive erythroid progenitors migrate on and within the fibroblastoid stroma to form large colonies of hemoglobinized erythroblasts that proceed to enucleate. Surprisingly, most (usually >95%) of the colonies that form in this culture system are large erythroid burst colonies. Very few granulocyte-macrophage (GM) colonies are found. This is in sharp contrast to the fact that the input bone marrow cell preparation has roughly equal numbers of primitive erythroid progenitors (BFU-E) and myeloid progenitors (CFU-G/M) and that nearly 100% of the mature cells produced in long-term liquid culture are granulocytes and macrophages, even in the presence of erythropoietin.[13,14]

Three mechanisms can be proposed to account for the predominant erythroid development in the coverslip-transfer culture system: (a) Both BFU-E and CFU-GM bind to the stromal cells, although stromal fibroblastoid cells, through either the production of growth factor or other unknown mechanisms, stimulate the growth of BFU-E preferentially; (b) BFU-E bind to the stromal cells with a greater affinity than do CFU-GM; (c) and contact between CFU-GM and fibroblastoid cells inhibits their development. This is unlikely, however, because CFU-GM develop successfully on the stromal layer in long-term bone marrow culture. Moreover, direct overlay of progenitors on stromal fibroblastoid cells (ie, without the adhesion and washing procedure to select for

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adherent progenitors) has been used successfully to enumerate CFU-GM in mouse bone marrow.[15] In this report, we provide evidence that the predominant erythroid development in this new culture system is due to the differential binding of BFU-E and CFU-GM to the stromal fibroblastoid cells. Furthermore, this binding is mediated by fibronectin produced by the stromal cells.

MATERIALS AND METHODS

Preparation of human bone marrow cells enriched for hemopoietic progenitors. Bone marrow mononuclear cells were collected from the interface after Ficoll-Hypaque gradient centrifugation, washed three times and resuspended in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 20% FCS. Monocytes were removed by overnight adherence to 60-mm Lux culture dishes (Miles Laboratories, Naperville, IL) at 37°C. Nonadherent marrow cells were collected, centrifuged, and incubated for 30 minutes at 4°C with predetermined optimal concentrations of a panel of monoclonal antibodies directed against myeloid, erythroid, and lymphoid maturation antigens. These antibodies included (specificity in parentheses): Leu 1 and Leu 5b (T cell), Leu 12 (B cell), Leu M1 (monocyte, granulocyte), Leu M3 (monocyte; all from Becton Dickinson), TG1 (myeloid maturation antigens, from Dr P. Beverly), Mo 1 and My 8 (monocyte and myeloid maturation antigens, both from Dr J.D. Griffin), and LICR.LON.R18 (glycophorin A, from Dr P. Edwards). After being washed to remove unbound antibodies, the cells were resuspended in phosphate-buffered saline (PBS)/5% FCS and incubated for 1 hour at 4°C in tissue culture dishes coated with rabbit anti-mouse immunoglobulin antibodies. The unbound marrow cells were removed by gentle swirling and tilting of the culture dishes, and by being washed twice in a similar manner with 5 mL of PBS/5% FCS. Usually 1% to 3% of the recovered cells were BFU-E and ∼1% to 3% were CFU-GM.

Coverslip-transfer culture using a preformed stromal cell monolayer and enriched bone marrow progenitors. The isolation and characterization of the human stromal cell strain ST-1 has been described.[16] The ST-1 cells were established from a long-term liquid culture of human fetal liver mononuclear cells. They are adherent, diploid, fibroblastoid in morphology, and synthesize collagen type I and fibronectin. They are usually maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 20% FCS.

Details of the coverslip-transfer culture have been published.[12] In brief, ST-1 cells of 18-21 passages were cultured as a monolayer on round glass coverslips with a diameter of 12 mm (Fisher Scientific, Pittsburgh). Ten coverslips with ST-1 cell monolayers were placed on the bottom of a 60-mm dish; 4×10^5 enriched bone marrow cells suspended in 4 mL of IMDM were added to the 60-mm dish containing the coverslips. The cultures were incubated at 37°C, 5% CO_2 for 2 hours. The coverslips were rinsed with warm IMDM to remove all unbound marrow cells. Each coverslip was then transferred to a 35-mm culture dish and overlaid with 3 mL of methylcellulose culture medium containing 10^{-6} mol/L of hydrocortisone and erythropoietin (Epo) (Terry Fox Laboratory, Vancouver, BC) at 2 U/mL. No other exogenous CSFs were added, since we have demonstrated that the ST-1–conditioned medium can support the development of both erythroid and GM colonies.[16] The cultures were incubated at 37°C in a high-humidity incubator with 5% CO_2. Colonies were scored on the tenth day. The procedure is illustrated in Fig 1.

Inhibition of the binding of enriched bone marrow cells to fibroblasts by anti-fibronectin antibodies. Rabbit anti-human plasma fibronectin antiserum (Calbiochem, LaJolla, CA) and the anti-HLA monoclonal antibody W6/32 (a gift from Dr S. Mentzer) were diluted with IMDM to the indicated concentrations and added to the coverslips with the ST-1 cell monolayers. After incubation at 37°C for 50 minutes, the coverslips were washed once with warm IMDM to remove unbound antibodies and used in the coverslip-transfer culture described above. The rabbit anti-human plasma fibronectin antiserum is specific for various epitopes on the fibronectin molecules. The monoclonal antibody W6/32 recognizes a monomorphic determinant on the α chain of all HLA-A, -B, and -C antigens. The antibody has a very high titer and blocks completely cytotoxic T lymphocyte-mediated cytotoxicity at 1:800 dilution.[17,18]

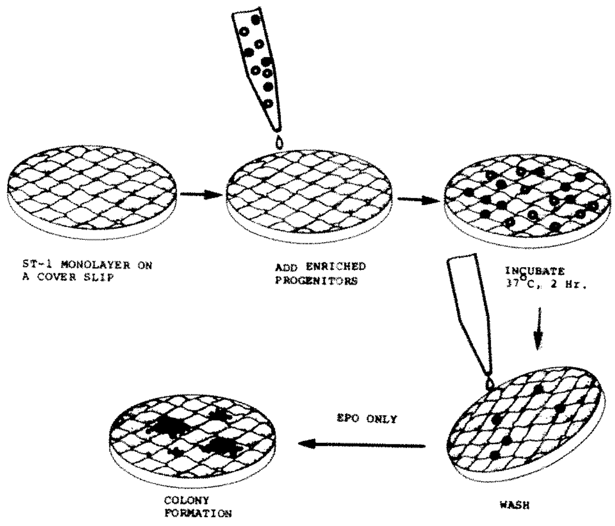

Fig 1. Outline of coverslip-transfer culture technique. Monolayers of human stromal fibroblasts (ST-1 cells) grown on 12-mm round glass coverslips are coincubated with enriched human bone marrow progenitors in serum-free culture medium for 2 hours at 37°C. Nonadhering bone marrow cells were washed away with warm serum-free medium. Each coverslip was then transferred to a 35-mm dish and overlaid with methylcellulose culture medium containing human erythropoietin (2 U/mL). Cultures were incubated at 37°C in a high-humidity incubator with 5% CO_2. Colonies were scored on day 10 to day 12.

Preparation of coverslips and Petri dishes coated with human plasma fibronectin. Human plasma fibronectin was affinity-purified using gelatin-Sepharose 4B (Pharmacia, Piscataway, NJ) from fresh citrated human peripheral blood according to the procedure of Ruoslahti and colleagues.[18] Five 12-mm round glass coverslips were placed on the bottom of a 35-mm tissue culture dish and coated with 2 mL of fibronectin solution (45 µg/mL; diluted in PBS, pH 7.5) at room temperature for 4 hours. Immediately before use, the coated coverslips were rinsed once with PBS to remove unbound fibronectin. Alternatively, 35-mm Petri dishes were coated with various concentrations of fibronectin and washed in the same manner. As a negative control, radioimmunoassay (RIA) grade bovine serum albumin (BSA; Sigma, St Louis) was diluted in PBS (45 µg/mL) and used to coat glass coverslips or 35-mm Petri dishes.

Coverslip-transfer cultures using fibronectin-coated coverslips and Petri dishes. Enriched human bone marrow cells 1×10^5 2 mL of IMDM were added to tissue culture dishes containing the coverslips or to fibronectin-coated Petri dishes. After a 2-hour coincubation at 37°C, the nonadherent marrow cells were washed away with warm IMDM. Individual coverslips were transferred to a 35-mm dish and overlaid with 3 mL of standard methylcellulose culture medium containing Mo cell-conditioned medium (10%) and erythropoietin (2 U/mL). Petri dishes were overlaid with the same culture medium after removal of the nonadherent bone marrow cells.

Erythroid colony-forming units (CFU-E) were counted on day 7, and BFU-E and CFU-GM were counted on day 14.

Inhibition of binding of progenitors to fibronectin-coated coverslips by domain-specific anti-fibronectin antibodies. The type 2 anti-human plasma fibronectin monoclonal antibody (Calbiochem) recognizes the cell-binding domain of the fibronectin molecule, whereas the type 3 anti-human plasma fibronectin monoclonal antibody (Calbiochem) recognizes the gelatin-binding domain of the fibronectin molecule.[19] All antibodies were diluted to the indicated concentrations and incubated with fibronectin-coated coverslips at 37°C for 50 minutes. After the incubation, excess antibodies were washed away with IMDM. The antibody-treated fibronectin-coated coverslips were used directly in coverslip-transfer culture experiments.

Immunofluorescence microscopy. Cultures were fixed in 3.7% formaldehyde in PBS (pH 7.4) at room temperature for 2 hours and permeated with cold (−20°C) methanol for 5 minutes. Residual formaldehyde was blocked by incubation with 1% BSA (Sigma) at 37°C for 30 minutes. Rabbit anti-spectrin IgG cross-reacted with both human and mouse RBC spectrins. Each coverslip was incubated with 20 μL of anti-spectrin IgG (1:200) for 30 minutes at 37°C in a humidity chamber. The coverslip was then overlaid with 20 μL of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200) and incubated for 30 minutes at 37°C in a humidity chamber. Stained coverslips were rinsed sequentially with PBS and distilled water and mounted in a gelatin/glycerol mixture. Immunofluorescence was carried out with a Zeiss photomicroscope equipped with epifluorescence.

Electronmicroscopy. Coverslip-transfer cultures were rinsed several times with warm IMDM (GIBCO) to remove methylcellulose, and fixed for 1 hour at room temperature in freshly prepared 2.5% glutaraldehyde 0.1 mol/L of cacodylate buffer. Fixed cultures were rinsed three times with 0.1 mol/L of cacodylate buffer, postfixed in 1% osmium tetroxide/1% potassium ferricyanide for 1 hour at 4°C. Postfixed specimens were rinsed three times with 0.1 mol/L cacodylate buffer, dehydrated in 30%, 50%, 70%, 95%, and finally 100% ethanol.

For scanning electronmicroscopy, fixed and dehydrated samples were critical point dried and sputter-coated with 100 Å gold palladium and examined with a JEOL 35CF scanning electron microscope.

Table 1. Addition of GM-CSF Does Not Increase Number of GM Colonies in Coverslip-Transfer Cultures

Surface Coating	Hemopoietic Growth Factors	BFU-E Colonies	CFU-GM Colonies	Erythroid Colonies (%)
	(Per coverslip; mean ± SEM, n = 5)			
BSA	Epo	0 ± 0	0.2 ± 0.4	—
BSA	Mo + Epo	0.2 ± 0.4	0 ± 0	—
ST-1	Epo	37.8 ± 2.5	1.4 ± 1.7	96.4
ST-1	Mo + Epo	38.8 ± 3.1	1.8 ± 1.5	95.6

Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; BSA, bovine serum albumin; ST-1, human stromal cells; Epo, erythropoietin; Mo, human lymphoblast cell line.

Round glass coverslips (12 mm) were coated with BSA (45 μg/mL) at room temperature for 4 hours. ST-1 cells were grown on glass coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS) and used before they reached 100% confluency. Both BSA and ST-1-coated coverslips were washed twice with DMEM immediately before the experiment and placed in the bottom of a 60-mm dish; 4 × 10^5 enriched human bone marrow cells were suspended in 4 mL of Iscove's modified Dulbecco's medium (IMDM), added to a 60-mm dish containing the coverslips, and coincubated for 4 hours at 37 °C. After coincubation, nonadherent bone marrow cells were washed away by rinsing the coverslips with warm IMDM three times. Individual coverslips were transferred to a 35-mm dish and overlaid with 3 mL of methylcellulose culture medium containing erythropoietin (2U/mL) or both erythropoietin (2U/mL) and Mo-conditioned medium (10% vol/vol). Colonies were scored 10 to 12 days after incubation. Standard short-term colony assay of the input bone marrow cells revealed 2,680 BFU-E and 1,680 CFU-GM per 10^6 cells plated (mean of duplicate).

RESULTS

Predominant erythroid development in the coverslip-transfer cultures is due to differential binding of BFU-E and CFU-GM to stromal fibroblasts. To distinguish between the two possible mechanisms (ie, differential stimulation *v* differential binding) that might account for the lack of GM colonies in the coverslip-transfer cultures, GM-CSF was added to the culture system in addition to erythropoietin. Any CFU-GM bound should then have been able to develop into GM colonies. In this study, we used the human lymphoblast cell line, Mo-conditioned medium, as a potent source of GM-CSF.[20] The result is shown in Table 1. The addition of exogenous GM-CSF did not increase or decrease the number of BFU-E- and CFU-GM-derived colonies, suggesting that the absence of development was not due to the lack of GM colony-stimulating activity (CSA). The results in Table 1 also show that the binding of BFU-E to stromal fibroblasts is a specific phenomenon, since BSA could not mediate such binding.

Binding of BFU-E to stromal fibroblasts can be blocked by anti-fibronectin antiserum. Because the ST-1 cells used in our coverslip-transfer culture system produce large amounts of fibronectin and since fibronectin has been shown to mediate the binding and migration of many cell types, we examined the effect of rabbit anti-fibronectin antiserum on the binding of BFU-E to stromal fibroblasts. The result is shown in Fig 2. The binding of BFU-E was inhibited by increasing concentrations of the anti-fibronectin antiserum. The inhibition varied from 70% to 99% in different experiments when the antiserum was used at a 1:20 dilution. Higher concentrations of the antiserum were not used because the fibroblasts began to show partial detachment from the coverslips, presumably due to the blocking effect of the anti-fibronectin antiserum. In contrast, the W6/32 monoclonal antibody specific for the α chain of all HLA-A, -B, and -C antigens did not affect the binding at all.

BFU-E bind to fibronectin-coated surfaces but CFU-GM do not. Petri dishes were incubated with increasing concentrations of fibronectin. Enriched bone marrow progenitors were then incubated on the fibronectin coated dishes for 2 hours, nonadherent cells were removed, and methyl cellulose culture mix was added to the plates. Very few CFU-GM-derived colonies formed, whereas $\sim$10% of the input CFU-E and BFU-E adhered to the dishes and formed colonies, with the plateau of maximal adherence occurring at $\sim$20 μg/mL (Fig 3).

We demonstrate a similar pattern of differential binding of BFU-E and CFU-GM to fibronectin by using glass coverslips coated with purified human plasma fibronectin (Table 2). Again, many more BFU-E than CFU-GM bound

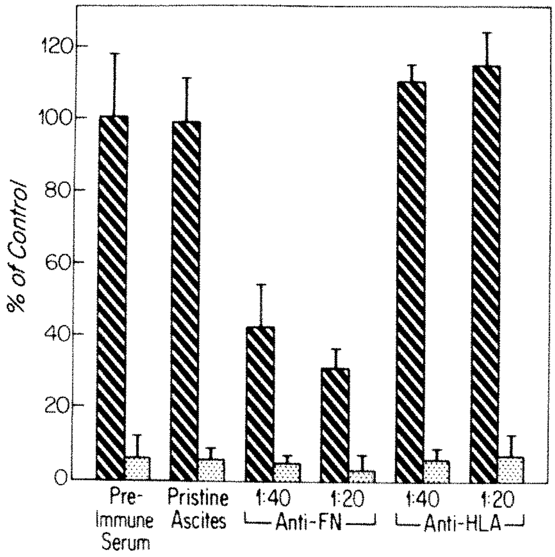

Fig 2. Inhibition of the binding of erythroid burst-forming units (BFU-E) to stromal fibroblasts (ST-1) by anti-fibronectin antiserum. Monolayers of ST-1 cells on coverslips were incubated with preimmune rabbit serum (1:20 dilution), pristine mouse ascites (1:20 dilution), rabbit anti-fibronectin antiserum (1:20 and 1:40 dilutions), or W6/32 monoclonal antibody (anti-HLA) (1:20 and 1:40 dilutions) for 50 minutes at 37°C. Unbound antibodies were washed away before the addition of enriched progenitors. Coverslip-transfer cultures were done as described in the Materials and Methods section. Colonies were scored on day 10. Hatched bars, erythroid burst colonies; dotted bars, granulocytes/macrophage (GM) colonies. Data are expressed as percentages of the number of erythroid burst colonies in the control with preimmune serum. Mean ± SEM; n = 5. Standard short-term colony assay of the input progenitor population received 420 BFU-E and 830 CFU-GM per 10^5 cells plated (mean of duplicate).

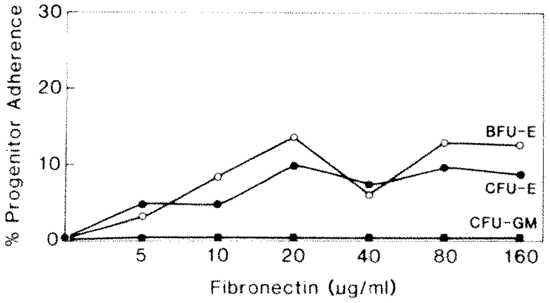

Fig 3. Adherence of BFU-E and CFU-E to purified fibronectin-coated Petri dishes; 35-mm Petri dishes were incubated with increasing concentrations of fibronectin for 2 hours and washed, and nonspecific binding sites were blocked by 15-minute incubation in bovine serum albumin (BSA). Enriched progenitors (3,340 BFU-E and 2,380 CFU-GM/10^5 cells) were incubated for 2 hours at 37°C in the coated Petri dishes, and nonadherent cells were removed by gentle washing. Progenitors were assayed in the nonadherent fraction and in the Petri dishes by overlaying with 2 mL of methylcellulose mix. Results were expressed as the percentage of adherent progenitors of the total (adherent and nonadherent) enumerated (in duplicate) at each concentration point. Increasing the concentration of fibronectin results in increased BFU-E and CFU-E binding, to a plateau of 13% and 10%, respectively, at 20 μg/mL. Very few CFU-GM bound to the fibronectin-coated surface, and very little binding of any progenitor class was observed when BSA alone was used to coat the dishes (data not shown).

Table 2. BFU-E but not CFU-G/M Binds to Fibronectin-Coated Surfaces

Type of Surface	Surface Coating	BFU-E Colonies	CFU-GM Colonies
		Per coverslip; Mean ± SEM, n = 5	
Glass coverslip	BSA	0.2 ± 0.4	0 ± 0
Glass coverslip	Fibronectin	57.2 ± 9.1	3.0 ± 2.2
		Per 35-mm Dish; Mean of Duplicate	
Petri dish	BSA	3.0	0.5
Petri dish	Fibronectin	≥200	6.0

Five round glass coverslips were coated with BSA or purified human plasma fibronectin at 45 μg/mL and placed (after coating) on the bottom of a 35-mm tissue-culture dish. The Petri dishes were coated with 2 mL of BSA or fibronectin solutions at 45 μg/mL for 4 hours at room temperature; 1 × 10^5 enriched human bone marrow progenitors in 2 mL of IMDM were added to each tissue-culture dish containing the BSA- or fibronectin-coated coverslips or to the coated Petri dishes. After a 2-hour coincubation, the nonadherent cells were washed away with warm IMDM. The coverslips were processed according to the method described in the footnote to Table 1. Each Petri dish was overlaid with 3 mL of methylcellulose culture medium. All cultures contained erythropoietin (2 U/mL) and Mo CM (10% vol/vol). Standard short-term colony assay of the input bone marrow cells revealed 2,680 BFU-E and 1,680 CFU-GM per 10^5 cells plated (mean of duplicate).

to fibronectin-coated glass coverslips; binding to Petri dishes is shown as a control.

BFU-E bind to the cell-binding domain on fibronectin. The fibronectin molecule has been shown to have separate domains for cell attachment, cell migration, collagen binding, and heparin binding.[9,19,21] To determine whether BFU-E bind to the same cell-binding domain on fibronectin as do fibroblasts, we used a mouse monoclonal antibody directed against the cell-binding domain of fibronectin.[19] The result in Fig 4 demonstrates that >90% of the binding was blocked by this antibody (type 2) at a final dilution of 1:40 or 1:20, providing evidence that this domain is involved in the binding of primitive erythroid progenitors to fibronectin. In contrast, the gelatin-binding domain-specific monoclonal antibody (type 3) did not inhibit the binding.

Erythroid progenitors remain associated with fibronectin until the perienucleation stage. Erythroid progenitors remain associated with the monolayer of stromal fibroblasts or the fibronectin-coated surface throughout differentiation: their association with fibronectin-coated coverslips until the enucleation stage is illustrated in Fig 5. After this stage, the erythroblasts become less tightly bound and can be easily detached. We also observed that the few GM colonies that developed in the coverslip-transfer cultures were usually suspended in the culture medium, not associated with the stromal fibroblasts or fibronectin-coated Petri dishes.

Immunofluorescent microscopy of differentiating erythroid progenitors stained with antispectrin IgG. Erythroid progenitors adhere preferentially to fibronectin-coated glass coverslips and migrate very slowly (observed by time-lapse video recording) in all directions at the early stages of their differentiation. The migrating cells have a

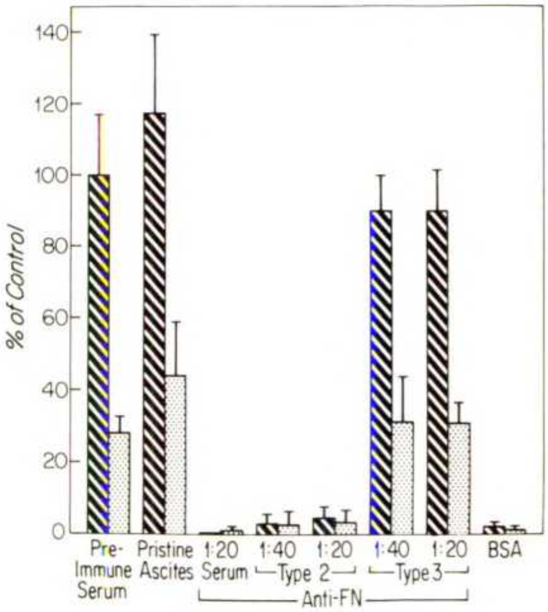

Fig 4. Inhibition of the binding of BFU-E to fibronectin by a monoclonal antibody specific for the cell-binding domain of fibronectin. The procedure was the same as described in the legend to Table 2 except that the fibronectin-coated coverslips were treated with different antibodies before the addition of enriched bone marrow cells. Rabbit anti-fibronectin antiserum (1:20) was used as a control. Type 2 monoclonal antibody specific for the cell-binding domain and type 3 monoclonal antibody specific for the gelatin-binding domain of fibronectin were used at 1:20 and 1:40 dilutions. Bovine serum albumin (BSA)-coated coverslips were included to demonstrate the specificity of fibronectin-mediated binding. Hatched bars, erythroid burst colonies; dotted bars, granulocyte/macrophage colonies. Data are expressed as percentages of the number of erythroid burst colonies in the control with preimmune serum. Mean ± SEM; n = 5. Standard short-term colony assay of the input progenitor population revealed 415 ± 44 BFU-E and 590 ± 74 CFU-GM per 10^5 cells plated (n = 4).

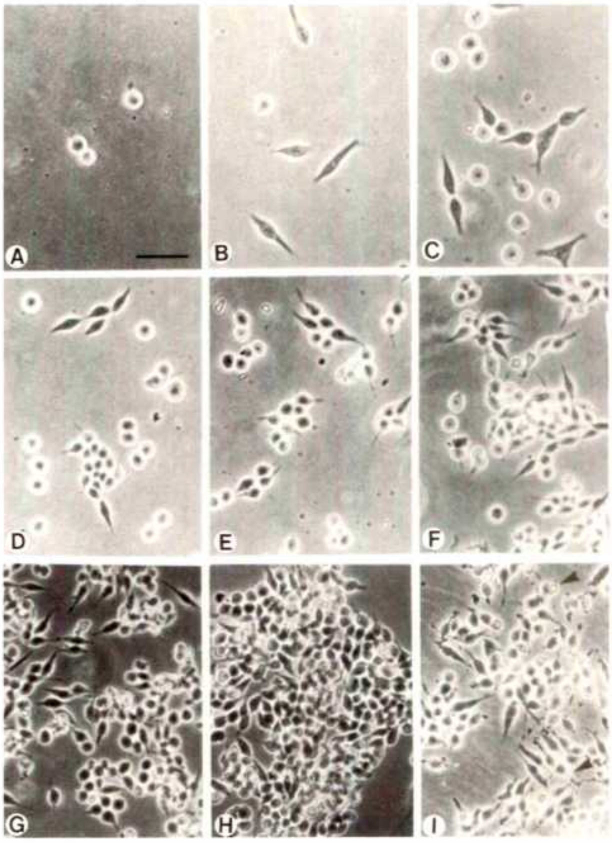

Fig 5. Serial phase-contrast photomicrographs of erythroid progenitors cultivated on glass coverslips coated with fibronectin. (A) day 1, (B) day 2, (C) day 3, (D) day 4, (E) day 5, (F) day 7, (G) day 8, (H) day 9, (I) day 10. Enucleating cells are evident (arrowheads), as is random migration and gradual decrease in cell size. Visible hemoglobinization began around day 8. All cells after day 8 were strongly positive for glycophorin A, spectrin, and dimethoxybenzidine stain (data not shown). Bar = 50 µm.

spindle shape (Fig 6A). All such cells are strongly positive for spectrin on day 5 in culture (Fig 6B). By day 10, as the progenitors begin to hemoglobinize, they gradually stop migrating. This is reflected in the decrease of the frequency of spindle-shaped (ie, migrating) cells (Fig 6C). The hemoglobinizing erythroblasts assume a spherical shape and are positive for spectrin (Fig 6D) and glycophorin A (data not shown). Hemoglobinized erythroblasts adhere less tightly to fibronectin and therefore frequently detach during the fixation and washing process, leaving fragments of cytoplasm and cell membrane on the fibronectin-coated coverslips (Fig 6C and E). Curiously, such fragments are usually negative for spectrin (Fig 6D and F). As the erythroblasts begin to enucleate (around day 12), the cytoplasm becomes very strongly positive for spectrin. Spectrin appears to be excluded from the thin rim of cytoplasm enveloping the partially expelled nuclei (Fig 6F).

Erythroid progenitors exhibit characteristic cell surface structures. Sequential scanning electron microscopy (SEM) of erythroid progenitors cultivated on fibronectin-coated surface from day 5 through day 12 revealed several cell surface features that appeared to be characteristic of erythroid progenitors (Fig 7). At the earlier stages of their differentiation (day 5 in culture), they exhibited many gyri and sulci on the main portion of the cell body. In addition, there were multiple microspikes, indicating their high motility at this stage of differentiation (Fig 7A). As they differentiated, the number of microspikes decreased, reflecting the decrease of motility (Fig 7B). After hemoglobinization, the number of gyri and sulci decreased, and the cell surface appeared smoother (Fig 7C). This trend continued until a smooth-surfaced reticulocyte was formed (Fig 7D and E).

DISCUSSION

In human adults, hemopoiesis occurs in the extravascular space between the marrow sinusoids. This extravascular space is composed of fibroblastoid adventitial reticular cells, macrophages, the extracellular matrix, and hemopoietic stem cells and their progeny in all stages of differentiation. The nonhemopoietic components of this complex structure are collectively called "marrow stroma" and are believed to provide the hemopoietic microenvironment required for the renewal and differentiation of hemopoietic stem cells.[22-24]

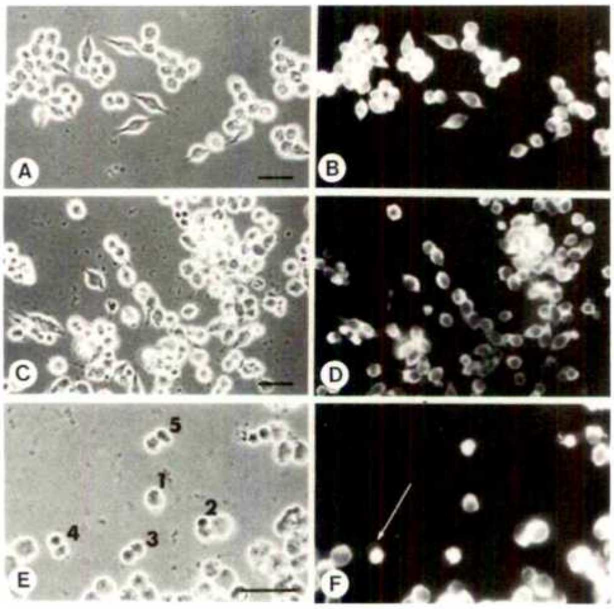

Fig 6. Phase-contrast and immunofluorescent microscopy of erythroid progenitors cultivated on fibronectin-coated glass coverslips. Cultures were fixed on day 5 (A,B), day 10 (C,D), or day 12 (E,F), permeated with cold methanol and stained with a rabbit anti-spectrin IgG, followed by FITC-conjugated goat anti-rabbit immunoglobulins. Phase contrast (A,C,E). Immunofluorescence (B,D,F). All cells were positive for cytoplasmic spectrin. Migrating cells appear as spindle-shaped cells. The debris in C and E are fragments of erythroblasts that detached during the washing and staining. Such fragments are usually negative for spectrin. E: Several cells at successive stages (1 through 5) of enucleation. The protruding nucleus had a smooth spherical surface and was negative for spectrin (arrow, F), whereas the cytoplasmic portion had a convoluted shape and was intensely positive for spectrin. Bar = 50 μm.

However, the hemopoietic microenvironment has never been defined in functional or molecular terms.

The long-term bone marrow liquid culture introduced by Dexter and colleagues has provided a useful in vitro culture system for the study of the stroma.[4,25,26] In this culture system, the proliferation and differentiation of hemopoietic stem cells can be maintained over a long period (weeks to months), provided that an adherent stromal layer is present. This adherent stromal layer is believed to possess the salient features of the bone marrow stroma in vivo.[24,27-29] However, only granulocytes and monocytes are produced.[14] The differentiation of erythroid progenitors is usually blocked at the stage of BFU-E, even with erythropoietin added.[13] Thus, it has been impossible to analyze the interactions between erythroid progenitors and the stromal cells in the long-term bone marrow liquid cultures.

Recently, we introduced a coverslip-transfer culture system designed to study the interactions between enriched hemopoietic progenitors and stromal cells.[12] In contrast to the long-term bone marrow culture, most of the colonies that develop in the coverslip-transfer culture system are erythroid. Their clonal origin is suggested by their discrete appearance on the stromal monolayers and also by time-lapse video recording. These data are not shown, but representative photomicrographs are presented in Fig 5. In this study, we provide evidence that human primitive erythroid progenitors bind to stromal fibroblasts and fibronectin with a greater affinity than granulocyte-macrophage progenitors. This finding seems to contradict many observations that erythroblasts are preferentially associated with macrophages in a structure called the "erythroblastic island," whereas granulocytes seem to be preferentially associated with adipocytes or fibroblastoid reticular cells in bone marrow,[30,31] or in vitro.[32,33] The blood cells described in those reports were "morphologically recognizable" erythroblasts or myeloblasts, however; both are rather differentiated "precursors," not the progenitors (morphologically unrecognizable) examined in the present study.

Our calculation indicates that roughly 10% to 16% of the BFU-E in the input population detected in the standard short-term colony assay were transferred along with the ST-1 monolayer or fibronectin-coated culture surface after the washing procedure. The explanation for the small proportion of erythroid progenitors bound to fibronectin is probably technical. When enriched bone marrow cells were serially transferred to three fibronectin-coated plates, a similar proportion of BFU-E (~10%) bound on each occasion (data not shown). This suggests that the incubation conditions may be inadequate for optimal exposure of all BFU-E to fibronectin molecules, perhaps due to the two-dimensional distribution of fibronectin on the culture plates. In vivo BFU-E would be exposed to fibronectin in three dimensions. Alternatively, BFU-E–derived colonies enumerated in short-term colony assay are heterogeneous in size and in colony morphology. They are probably derived from erythroid progenitors at various stages of differentiation, with the larger colonies being derived from the more primitive progenitors since they have a greater proliferative potential. In the present study, the erythroid progenitors transferred were those that bound most strongly (to withstand the washing and manipulation) to fibronectin. Because most of the transferred progenitors formed large erythroid colonies consisting of several thousand erythroblasts, and therefore were probably the more primitive ones, primitive erythroid progenitors may have bound more strongly to fibronectin than the more mature erythroid progenitors and were selectively transferred. Evidently, however, erythroid progenitors at all stages of differentiation do exhibit an affinity for fibronectin since differentiating erythroid progenitors and precursors remain bound to fibroblasts[12] and fibronectin-coated surfaces until enucleation (Fig 5).

That 70% to 99% of the binding of BFU-Es to fibroblasts could be blocked by rabbit anti-fibronectin antiserum indicates that most, if not all, of the binding was mediated by cell-surface fibronectin. The HLA α chain-specific monoclonal antibody W6/32 could not inhibit the binding. Furthermore, the binding between erythroid progenitors and fibronectin was inhibited by a monoclonal antibody specific for the cell-binding domain of the fibronectin molecule, but not by the monoclonal antibody specific for the gelatin-binding domain of fibronectin (Fig 3). Recently, both MEL cells and reticulocytes were shown to bind to the same cell-binding domain on fibronectin as do normal rat kidney (NRK) fibroblasts.[7,34] A 140-kd fibronectin receptor on MEL cells has been described. The gradual loss of this

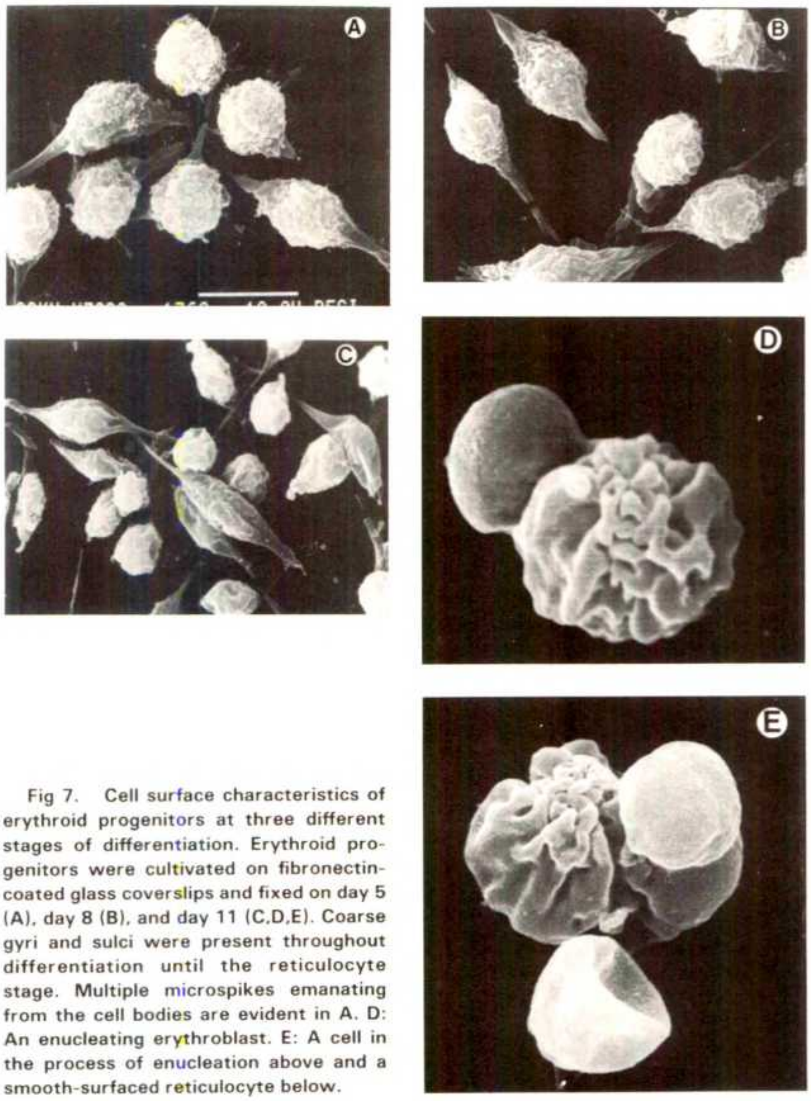

Fig 7. Cell surface characteristics of erythroid progenitors at three different stages of differentiation. Erythroid progenitors were cultivated on fibronectin-coated glass coverslips and fixed on day 5 (A), day 8 (B), and day 11 (C,D,E). Coarse gyri and sulci were present throughout differentiation until the reticulocyte stage. Multiple microspikes emanating from the cell bodies are evident in A. D: An enucleating erythroblast. E: A cell in the process of enucleation above and a smooth-surfaced reticulocyte below.

receptor on differentiation of MEL cells correlated with a loss of adhesion to fibronectin.[7] Taken together, these data suggest that erythroid progenitors bind to fibronectin throughout the differentiation process from the BFU-E stage to the reticulocyte stage, with gradual loss of binding toward the end of the differentiation. Myeloid progenitors, on the other hand, do not bind to fibronectin as strongly as do BFU-E; they may develop affinity for fibronectin as they differentiate and mature into functional granulocytes and macrophages. Other components of the extracellular matrix such as the proteoglycans are synthesized by many stromal cell types, including fibroblasts, endothelial cells, monocytes, and smooth muscle cells. Some of these components may play a role in myeloid cell adhesion and differentiation.[35-37]

Using this approach, we were able to study the surface characteristics and the ultrastructure of erythroid progenitors with relative ease. The earlier erythroid progenitors had many coarse gyri and deep sulci, resembling the surface of the cerebral hemisphere (Fig 7A). These gyri and sulci are different from the membrane ruffles or lamellipodia usually seen on fibroblasts or macrophages. Aside from the coarse gyri and sulci, progenitors possess multiple microspikes. We believe that these microspikes,[38] like those on fibroblasts or on the growth cone of neurites, are involved in the migration of erythroid progenitors. Indeed, we observed the slow and random migration of erythroid progenitors on fibronectin-coated surfaces using time-lapse video recording (data not shown). The migration ceases after the hemoglobinization of the erythroblasts, the stage when microspikes are no longer seen.

We propose that the stromal fibroblast/fibronectin represents a microenvironmental niche in which the primitive erythroid progenitors reside and migrate. The stromal fibroblasts provide both extracellular matrix and hemopoietic growth factors for the developing erythroid progenitors. As erythroid progenitors differentiate and mature into erythrocytes, they gradually lose affinity for fibronectin, dissociate from this particular niche, and enter the circulation.

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Predictive Markers of Chronic Liver Disease in Hemophilia

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In an attempt to predict progressive liver damage in hemophiliac patients by noninvasive means, we conducted a retrospective analysis of clinical and laboratory data from 44 liver biopsies taken from 35 hemophiliac patients. This showed that serum IgG was normal in patients with chronic persistent hepatitis (CPH) but significantly elevated in those with chronic active hepatitis (CAH) or cirrhosis (CIR) ($P < .001$). Relationships were less significant between liver histology and IgM ($P < .01$), IgA ($P < .05$), and globulin ($P < .05$). This was unaffected by human immunodeficiency virus (HIV) antibody status in asymptomatic individuals. Although patients with progressive liver disease were also older than those with CPH ($P < .001$), the immunoglobulin abnormalities were independent of this. Neither clinical examination nor liver biochemistry at the time of biopsy were of significant diagnostic value. Our results indicate that in the absence of AIDS an elevated IgG level is a reliable indicator of progressive hemophilic liver disease.
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RECENT LIVER biopsy data show that progressive liver disease is a serious problem in hemophilia[1-3] and may be the most common cause of death among hemophiliac patients.[4] Whereas most hemophiliac patients have minor degrees of liver disease such as chronic persistent hepatitis (CPH), a substantial proportion have chronic active hepatitis (CAH) or cirrhosis (CIR), usually without symptoms. Because these patients are at risk of developing clinical problems, early identification is important so that they may be followed and treated appropriately. Currently, liver biopsy is regarded as the only reliable method for distinguishing between nonprogressive hepatitis and more serious liver disease. Many clinicians are reluctant to resort to liver biopsy because of the inherent risks of the procedure; indeed, liver biopsy in hemophiliac patients has been suggested to be particularly hazardous.[2] Therefore, a simple and more readily available marker of severe hemophilic liver disease is needed. This prompted us to perform a retrospective analysis of clinical and laboratory data from our 35 biopsied hemophiliac patients to see whether any of these enabled us to distinguish patients with progressive liver disease from those with nonprogressive liver disease.

MATERIALS AND METHODS

Patients. Thirty-five patients underwent liver biopsy between 1977 and 1986. The selection criteria have been published elsewhere.[1] The mean age was 33.4 ± 18.2 years (Mean ± 1 SD) (3 to 70 years) at the time of their first biopsy. Thirty-two had hemophilia A, 2 had hemophilia B, and 1 had von Willebrand's disease. Twenty-four were severely affected (factor VIII or IX <2%), and 11 were mildly affected (factor VIII or IX >2%). Nine of these had a second liver biopsy after a mean interval of 46.7 ± 21 months (range 24 to 96 months). Factor VIII or IX consumption during the 3 years prior to each biopsy was calculated from the hospital records and cross-checked with the patient's factor VIII or IX returns.

Liver biopsies. All liver samples were obtained using a Klatskin needle with precautions as previously described.[5] Details of the histological processing have also been described before.[1] The presence of microvesicular steatosis, sinusoidal infiltration, and periductal infiltration was construed as evidence of non-A, non-B hepatitis virus infection (NANB).[6,7] All liver biopsies were stained, using an immunofluorescent method, for δ antigen.

Physical signs. All patients were seen every 6 months for blood sampling and physical examination. Over the past 4 years, this has been performed by the same physician each time.

Biochemistry. Serum aminotransferases, bilirubin, albumin, and globulin levels were measured using standard auto analyzer techniques. Prothrombin times were also measured at the time of biopsy, partly as a measure of hepatic function. All values outside the reference range were considered abnormal. When three or more consecutive aminotransferase levels from a patient, covering a period of at least 1 year, were all normal, the patient's aminotransferase pattern was considered normal. When three or more tests covering a 2-year period were all abnormal and there was no evidence of a return to normal, the patient's aminotransferase pattern was considered persistently abnormal. Patients with at least three observations over a 2-year period not conforming to either pattern were considered to have an intermittently abnormal aminotransferase pattern.

Serology. Serum immunoglobulin levels were determined using an immunonephelometric assay using endpoint kinetics on a rapid centrifugal auto-analyzer (Baker Instruments, Encore). Antinuclear, anti-smooth muscle, and antimitochondrial antibodies were measured by indirect immunofluorescence, screened at a titer of 1 in 20; anti-dsDNA was measured by reverse passive hemagglutination.

Hepatitis B serology including HBsAg, anti-HBc, and anti-HBs were determined by radioimmunoassay (RIA) at the time of biopsy. IgM anti-HBc was also determined, using RIA, in patients who had no evidence of anti-HBs following a documented episode of hepatitis B, to exclude continuing active hepatitis B infection.

Human immunodeficiency virus (HIV) antibody was measured by RIA in stored serum samples taken serially between 1977 and 1986. The relationship between serum IgG level and HIV seroconversion and between serum IgG level was analyzed in 23 HIV antibody-positive hemophiliac patients, 15 of whom had undergone liver biopsy.

Statistics. Dividing the patients into those with CPH, those with CAH, and those with CIR, we analyzed the relationship between histological diagnosis and various parameters of possible predictive value. The relationship between liver histology and age, factor VIII or IX consumption in the 3 years prior to biopsy, and mean serum immunoglobulin and globulin levels during the year of biopsy was examined by analysis of variance. The relationship between liver histology and the degree of abnormality of the aminotransferases during the year of biopsy was analyzed using one-way nonparametric Kruskall-Wallis analysis of variance. Discriminant analysis was

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Table 1. Results of Serial Liver Biopsies

Patient	1	2	3	4	5	6	7	8	9	10	11	12
First biopsy	CPH	CPH	CPH	CPH	CPH	CPH	CPH	CPH	CAH	CAH	CAH	CAH
Second biposy	CPH	CPH	CAH	CAH	CIR	CIR	CIR*	CIR*	CIR*	CPH	CIR	CIR

Abbreviations: CPH, chronic persistent hepatitis; CAH, chronic active hepatitis; CIR, cirrhosis.

*Not rebiopsied; clinical evidence of CIR including esophageal varices.

applied to combinations of mean IgG, IgM, and IgA levels during the year of biopsy in an attempt to improve the predictive power of IgG alone.

Because there was a significant positive correlation between progressive liver disease and both increased serum IgG and increasing age, it was important to establish whether serum IgG varied independently of age. Therefore, since CPH is not in itself associated with a raised IgG level,[8] the relationship between IgG and age was analyzed in 24 hemophiliac patients, aged 3 to 66 years with biopsy-proven CPH. Using Kendall's rank correlation coefficient, all 137 IgG estimations from these patients were analyzed in relation to age at the time of sampling. The relationship between serum IgG level during the year of biopsy and the factor VIII consumption during the 3 years prior to biopsy was analyzed using Pearson's product-moment correlation coefficient for the 39 biopsies for which this information was available. The change in serum IgG level at the time of HIV antibody seroconversion was analyzed using Student's paired t test.

RESULTS

Initial biopsy in 35 patients showed CPH in 21, CAH in 9, and CIR in 5. Nine patients underwent a second biopsy, results of which are shown in Table 1. A further three patients developed esophageal varices and other physical signs of CIR (Table 1). Therefore, of the 44 liver samples from 35 patients, 24 had CPH, 11 had CAH, and 9 had CIR, and there is now clinical evidence of CIR in 3 other patients.

Histological stigmata suggestive of NANB hepatitis were found in 33 biopsies from 27 patients (20 CPH, 7 CAH, 6 cirrhosis). None of the liver samples had ground-glass or orcein-staining hepatocytes indicative of continuing intrahepatic HBV infection. HBsAg was not detected in the serum of any patient at the time of biopsy. Continuing HBV infection was suggested in one patient with CPH, who was biopsied 6 months after an episode of typical hepatitis B, by the presence of IgM anti-HBc.

Physical signs. Four patients, all with cirrhosis, had spider naevi. Eight patients had hepatomegaly (2 had CAH and 6 had CIR) at the time of biopsy. The spleen was palpable in 6 (in 3 with CPH, in 3 with CIR).

During a mean period of observation of 48 ± 26 months after biopsy, the spleen became palpable in 9 other patients (3 with CPH, 3 with CIR and 3 with clinical CIR). Thus, at the time of writing, 15 patients have a palpable spleen; 8 of these are now HIV antibody-positive (3 with CPH, 5 with CIR). Esophageal varices were demonstrable in only 3 of the 8 HIV-positive patients. Although 9 of our 12 patients with CIR have splenomegaly, esophageal varices were demonstrable in only 6. Three patients with CIR developed ascites and hepatic encephalopathy. All three died, two of hepatic failure and the third of intracranial haemorrhage. The number of patients with abnormal physical signs was too small for statistical analysis.

Age at time of biopsy. Age at the time of biopsy (44 biopsies) differed significantly among the three groups of patients with CPH, CAH, and CIR; their mean ages were 28.2, 33.7, and 53.4 years, respectively ($P < .001$). The age of those with CIR was also significantly higher than those with CAH and those with CPH ($P < .05$).

Liver biochemistry. We could demonstrate no statistically significant relationship between the mean degree of elevation of the AST or ALT during the year of biopsy and the liver histology (Table 2). Serum bilirubin, serum albumin, and prothrombin time were invariably normal during the year of biopsy, but became abnormal during the last few months of life in the three patients who died. Mean serum globulin level was significantly higher in those with CIR ($P < .05$) than in those with CPH or CAH.

Serum immunoglobulins. Simultaneous immunoglobulin data were available for 41 of the 44 biopsies. There was a highly significant difference in mean IgG level during the year of biopsy among the three histological groups ($P < .001$), using the analysis of variance (Fig 1). The mean IgG level in the group with CIR was significantly higher than in both the CAH and the CPH group ($P < .05$). Similarly, the IgG level in the CAH group was significantly higher than in the group with CPH ($P < .05$). Patients with CPH had serum IgG levels consistently within the normal range, whereas IgG level did not reliably discriminate between CAH and CIR; the higher the serum IgG level, the more likely the presence of CIR. Discriminant analysis of combinations of IgG, IgM and IgA results failed to improve on the predictive value of IgG alone. Among the nine patients who underwent a second liver biopsy, progressive liver disease in six patients was associated with a rise in the mean IgG during the year of biopsy from 13.8 ± 2.4 g/L at the time of the first biopsy to 21.1 ± 8 g/L at the time of the second biopsy. Similar changes were observed in two patients with clinical evidence of progression to CIR in whom serial immunoglobulin levels were available. In contrast, in the three patients in whom the liver disease remained static or improved, IgG levels remained unchanged and within the normal range (Fig 2), but the number of observations was too small for statistical analysis.

Table 2. Mean AST and ALT in U/L During the Year of Biopsy in Relation to Biopsy Result

	CPH (n = 25)	CAH (n = 10)	Cirrhosis (n = 9)
AST (7-40 U/L)	72 ± 48	143.2 ± 126	89.1 ± 40.8
Range	23-137	52-448	33-167
ALT (7-45 U/L)	131.6 ± 95.4	197 ± 109	116 ± 94
Range	22-407	77-420	53-372

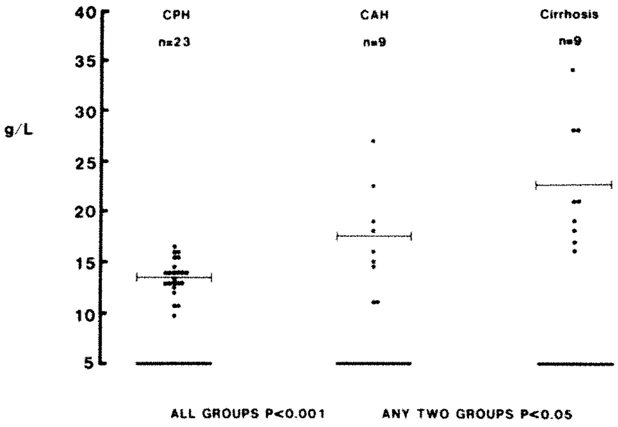

Fig 1. Relationship between IgG and liver histology for 41 liver biopsies; normal range 5.3 to 16.5 g/L.

No relationship between factor VIII use in the 3 years prior to biopsy and serum IgG level could be demonstrated for the 39 biopsied patients for whom this information was available ($r = -.1408$, $P = .20$). Similarly, no significant relationship could be demonstrated between serum IgG level and age, using 137 observations from 24 patients with CPH ($r = .270$, $P = .448$).

Serum IgM levels varied significantly among the three groups ($P < .01$), using the analysis of variance. The mean IgM in the group with CIR was significantly higher than that in the group with CPH ($P < .05$) but not higher than that in the group with CAH, indicating limited predictive power. Although IgA levels varied significantly between the three groups ($P < .05$), and was highest in those with CIR, no significant difference between any two groups could be demonstrated.

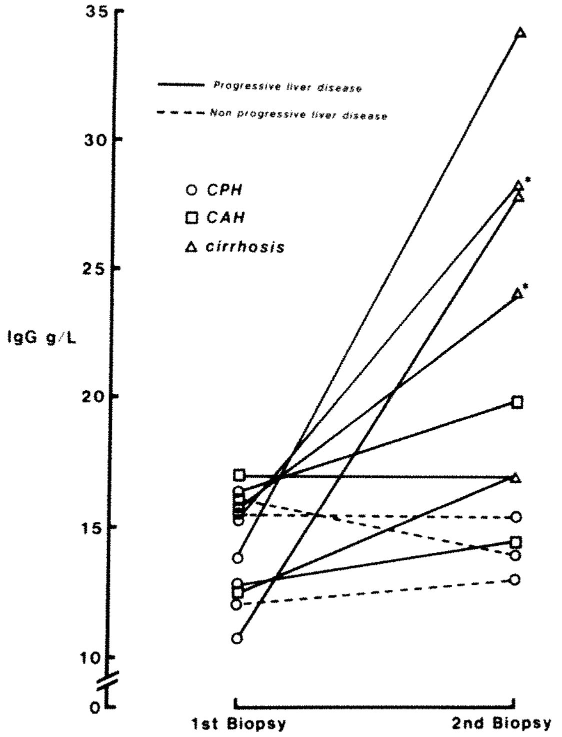

Fig 2. Change in IgG with both progressive and nonprogressive liver disease. *Clinical, not histological, evidence of cirrhosis and therefore not included in the analysis.

At the time of biopsy, only four of our patients were HIV antibody positive, and all had CPH and normal IgG levels. To determine the extent to which HIV virus infection might mask the relationship between IgG and liver disease, we studied 23 asymptomatic HIV antibody-positive hemophiliac patients, including 16 with persistently abnormal, 6 with intermittently abnormal, and 1 with normal aminotransferase levels. There was no significant difference between the mean IgG in the year before HIV seroconversion as compared with the year following HIV seroconversion of 16.34 ± 3.4 g/L *v* 15.9 ± 3.4 g/L.

Autoantibodies. Autoantibodies were repeatedly absent from all patients.

Factor VIII treatment. No significant relationship appeared to exist between the factor VIII consumption in U/kg per year during the 3 years prior to biopsy and the histological diagnosis ($P > .05$). Although the group with cirrhosis had the lowest factor VIII consumption, presumably because 5 of the 10 were mildly affected by hemophilia, this difference did not achieve statistical significance.

DISCUSSION

Despite our extensive experience of liver biopsy in hemophiliac patients without major complications, we were nevertheless concerned by the inherent risks of the procedure. This prompted us to analyze retrospectively the clinical and laboratory data from our 35 biopsied haemophiliac patients to see whether any of these were associated with progressive liver disease.

The presence of abnormal physical signs was of diagnostic value in only a few patients. Although patients with spider nevi always had CIR, these were found in only 4 of the 12 patients with this complication. Splenomegaly correlated poorly with portal hypertension, since esophageal varices were demonstrated in only six of the nine patients with CIR and splenomegaly. Indeed, the discovery of a palpable spleen in the absence of esophageal varices in five HIV antibody-positive patients suggests that splenomegaly may be a feature of HIV infection, as suggested by Ludlam and colleagues.[9] Splenomegaly was reported in hemophiliac patients even before the advent of the HIV infection, however.[10]

We confirmed earlier observations that the degree of elevation of the aminotransferases is of no diagnostic value.[3,11] Marginal elevations are found in all groups, and resolution of the biochemical abnormalities is observed in some subjects with developing CIR. The emergence of an intermittently abnormal pattern of aminotransferases in eight patients with CPH is consistent with the suggestion that CPH may be associated with this pattern of liver enzyme abnormality.[8,12]

The incidence of progressive liver disease increased significantly with advancing age. This may reflect the greater time that older patients have had for their liver disease to develop. Alternatively, it may suggest a change in host response to hepatotropic viruses with increasing age. It is unlikely to be accounted for by an increased total lifetime exposure to clotting factor concentrates, because we could find no relationship between the severity of the liver disease and the factor VIII consumption and because almost all patients develop NANB hepatitis after their first exposure to non-heat-treated factor VIII concentrate.[13]

Although it has been suggested that chronic HBV or δ infections are responsible for most cases of progressive hemophilic liver disease,[14,15] we can demonstrate no such clear-cut relationship. Serological evidence of continuing HBV infection was found in only one biopsied patient, who had CPH. This is representative of our population, since only 4 of our 150 hemophiliac patients are chronic HBV carriers. Similarly, δ infection was demonstrated in only six biopsied patients, all of whom have CPH. In contrast, 27 of the 35 biopsied patients have histological stigmata suggestive of NANB hepatitis. Therefore, having excluded other causes for liver disease, such as alcohol, HBV, and autoimmune disease, we believe that our biopsy group is etiologically a remarkably homogeneous one, in which most of the liver disease can be attributed to NANB hepatitis and very little to HBV or δ.

Hypergammaglobulinemia has been associated with CIR and CAH for many years,[16] although we are unaware of any reports of it in chronic NANB hepatitis. Although serum globulin, IgM, and IgA levels were significantly increased in patients with progressive haemophilic liver disease, they did not discriminate reliably between the various histologic groups. In contrast, the serum IgG level appeared to be a reliable indicator of both progressive and nonprogressive hemophilic liver disease. The serum IgG levels in patients with CPH were tightly grouped within the normal range, confirming the observations of Chadwick and colleagues in CPH following hepatitis B.[8] Although serum IgG did not reliably discriminate between CAH and CIR, serum IgG was significantly more elevated in patients with CIR than in those with CAH, and as the serum IgG level became higher, cirrhosis became progressively more likely. Progression of liver disease was almost invariably associated with a substantial increase in serum IgG. In contrast, serum IgG remained unchanged in three patients in whom no change occurred between biopsies (Fig 2). There is no relationship between age and IgG in our hemophiliac patients or indeed in normal subjects.[17] All patients in our series with serum IgG levels >17 g/L had CAH or CIR. Twenty-five of 106 hemophiliac patients seen in 1986 have IgG levels in this range, suggesting that the prevalence of CAH and CIR among hemophiliac patients treated with blood products is ~25%. This is in close agreement with the report of a retrospective study of 155 unselected liver biopsy and necropsy specimens by Aledort and colleagues.[2] CPH was observed to progress to CAH or cirrhosis in six of our patients and is clearly less benign than had earlier been supposed.[1] In view of this, serum IgG should be measured at intervals in such patients to enable one to identify those developing progressive liver disease.

Although 50% of cases of AIDS-related complex (ARC)[18] and most cases of AIDS[19] have elevated IgG and IgA levels, our results are unaffected by this since none of our patients suffered with either disorder at the time of biopsy. Moreover, only four individuals, all with CPH and normal IgG levels, were HIV antibody positive at the time of biopsy. HIV antibody seroconversion per se was also unassociated with any change in IgG level, consistent with reports of normal immunoglobulin levels in most asymptomatic HIV antibody positive patients from other risk groups.[18-20] We therefore feel confident that in the absence of AIDS, ARC, or autoimmune disease an elevated serum IgG level is a reliable marker of severe hemophilic liver disease. This requires prospective confirmation by others.

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Plasminogen Activator in Normal Subjects After Exercise and Venous Occlusion: t-PA Circulates as Complexes With C1-Inhibitor and PAI-1

By N.A. Booth, E. Walker, R. Maughan, and B. Bennett

Exercise to exhaustion was associated with the appearance in plasma of plasminogen activator (PA) in several mol wt forms, as identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with zymography. A number of active bands, all immunologically identified as tissue-type PA (t-PA), were observed. The major form had an apparent mol wt of ~60,000 and is due to free t-PA. The other strong bands had apparent mol wts of ~110,000 and 180,000. The 110,000 band, also present in pre-exercise samples, represents t-PA complexed with its major inhibitor (PAI-1), and the 180,000 band is due to t-PA complexed with C1 inhibitor. The released forms of t-PA were cleared rapidly after cesssation of exercise at exhaustion. Urokinase-type PA (u-PA) activity was also identified in pre- and postexercise samples at an apparent mol wt of ~50,000. This is consistent with its being free u-PA; no complexed forms of u-PA were observed. Qualitatively similar changes in plasma PA were observed after venous occlusion. Small quantities of plasmin were generated after strenuous exercise, as observed by detection of plasmin-α_2-antiplasmin complex by two-dimensional immunoelectrophoresis in three of five subjects. This complex was cleared rapidly after cessation of exercise. Plasmin-α_2-antiplasmin complex was not detected in any of the subjects after venous occlusion.

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FIBRINOLYTIC processes depend on the conversion of the plasma protein plasminogen to the protease plasmin by plasminogen activators. Two principal species of plasminogen activator (PA) have been identified, tissue-type PA (t-PA), identified in and purified from tissue extracts,[1] and urokinase-type (u-PA), purified from urine.[2] Both types of PA have been identified in human blood,[3,4] but their relative importance and physiological roles remain uncertain.

For many years, strenuous short-term or sustained physical exercise[5-8] and venous occlusion[7] have been known to increase plasma fibrinolytic activity; this rise reflects increased levels of plasminogen activator, primarily t-PA,[3,9] rather than formed plasmin. Numerous studies have confirmed these observations; most have used techniques such as the euglobulin lysis time (ELT) or fibrin plate lysis. Such methods, while primarily measuring overall PA activity, are materially influenced by other variables such as fibrinogen or plasminogen concentrations and by inhibitors of plasminogen activation or of plasmin itself.

We applied the technique of Granelli-Piperno and Reich[10] to analysis of samples of whole plasma.[11,12] This method uses sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate plasma proteins on the basis of molecular weight. Thereafter, PAs are identified zymographically by laying the washed gel on a detector gel that contains fibrin and plasminogen. PA activity is revealed after incubation by the development of visible bands of lysis in the opaque fibrin film in areas where PA has diffused into the detector gel and converted its plasminogen to plasmin with resultant local lysis of the fibrin. This technique separates PAs in plasma from one another, from plasminogen, and from the inhibitors of fibrinolysis. This allows their enumeration and characterization, in terms of their mol wt and immunological reactivity. This approach was used to study the response to strenuous standardized physical activity[13] and to venous occlusion to investigate the contribution of the different species of PA.

MATERIALS AND METHODS

Blood samples were collected into 0.1 vol of 0.13 mol/L of sodium citrate and chilled on ice. Platelet-poor plasma was prepared by centrifugation at 1,000 g for 15 minutes at 4°C. All plasma samples were stored at −70°C.

ELT and fibrin plate lysis assays were performed as previously described.[14] Fibrin plate assays were used to examine PA activity in both unfractionated plasma and in the euglobulin fraction. Plasmin-α_2-antiplasmin complexes in plasma were detected by the two-dimensional immunoelectrophoresis technique described previously.[15,16]

SDS-PAGE with zymography to detect PA was performed on plasma samples as previously described.[12] Immunological identification of the PA was achieved by inclusion of specific immunoglobulin to either t-PA or u-PA in the detector gels.[12] Antibodies to t-PA and u-PA were from Biopool (Hornefors, Sweden) and all other antisera were from Dako Immunochemicals, Copenhagen. Immunoblotting was performed as described by Burnette.[17] Immunodepletion was performed on columns of immunoglobulin linked to Sepharose 4B, as described previously.[18] C1-Inhibitor was kindly donated by Dr M. Smith, Department of Pathology, University of Dundee, Scotland.

Subjects: Physical exercise. Healthy normal male subjects between the ages of 21 and 32 years were studied. Five subjects (VO^{2max} 54, 58, 59, 60, and 62 mL/min/kg) were exercised to exhaustion on a bicycle ergometer at ~70% of their $VO^{2\,max}$. Venous blood samples were withdrawn immediately prior to exercise, at intervals up to exhaustion, and for several hours postexhaustion.

Subjects: Venous occlusion. Healthy men and women aged 26 to 45 years were studied. After a 30-minute period of recumbent rest, a venous blood sample was obtained from one arm. A sphygmomanometer cuff was applied to the opposite arm, and the pressure was maintained at midway between systolic and diastolic (usually 90 to 100 mm Hg) for 15 minutes, after which further blood samples were obtained from both arms and the pressure in the cuff was released.

All subjects for both exercise and venous occlusion experiments gave informed consent in accordance with the Helsinki Agreement on Human Experimentation.

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RESULTS

Zymography on plasma from normal resting subjects. SDS-PAGE with zymography on plasma samples from resting subjects produces two bands of plasminogen-dependent lysis, one of mol wt 110,000 and a faint one of 50,000, as well as a weak band of plasminogen-independent lysis of mol wt 95,000 (Fig 1). Inclusion of specific immunoglobulins to t-PA in the detector gel abolished the 110,000 band whereas immunoglobulin to u-PA abolished the faint 50,000 band. Neither had any effect on the plasminogen-independent activity (Fig 1).

Plasma from subjects after exercise or venous occlusion. Exercise or venous occlusion was associated with several changes in active bands of PA. A very broad band of PA of mol wt 55,000 to 65,000 appeared, sharply demarcated at its advancing border. The intensity of the band of 110,000 mol wt increased, and heterogeneity in this mol wt range appeared. A new band of activity of about 180,000 mol wt was observed.

Characterization of the PA activity appearing after exercise. The PA activity of plasma samples taken at exhaustion was examined using antibodies to t-PA and u-PA. Almost all the new bands of PA activity appearing in plasma after exercise were inhibited by antibody to t-PA (Fig 2). The exception was a crescent at the leading edge of the broad band of PA activity of ~60,000 mol wt that remained unaffected by this antiserum. This may be due to u-PA. Plasminogen-independent lysis did not appear to be affected by exercise (Fig 2). All bands identified in euglobulin fractions were inhibited by antisera to t-PA (Fig 3). Thus, no u-PA–related activity appeared in euglobulin precipitates.

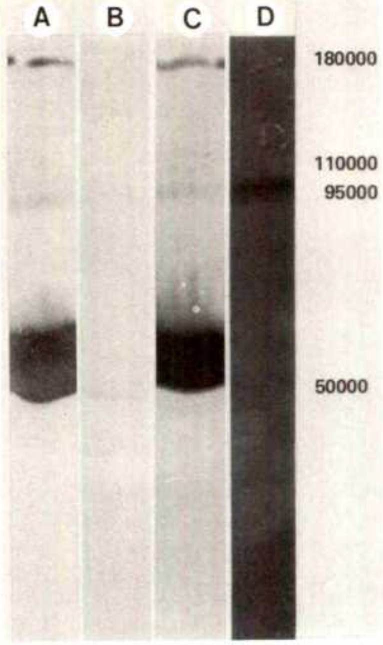


Fig 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with zymography of plasma taken after 80-minute exercise (exhaustion) (A) without immunoglobulin, (B) with immunoglobulin to tissue plasminogen activator (t-PA), (C) with immunoglobulin to urokinase plasminogen activator (u-PA), and (D) without plasminogen in the detector gel.

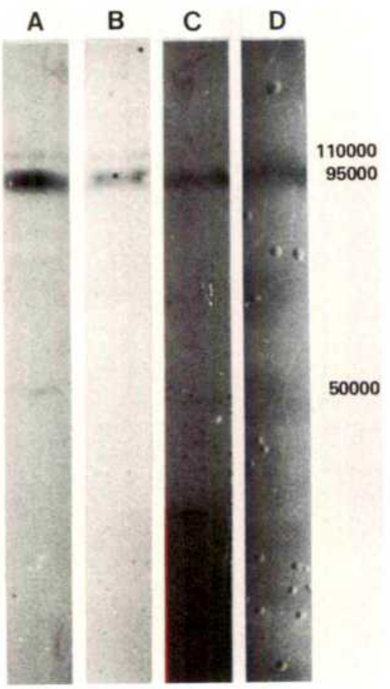


Fig 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with zymography of resting plasma (A) without immunoglobulin, (B) with immunoglobulin to urokinase plasminogen activator (u-PA), (C) with immunoglobulin to tissue plasminogen activator (t-PA) and (D) without plasminogen in the detector gel.

The t-PA in normal plasma is due to a complex of t-PA with its major inhibitor in the blood, PAI-1, and appears at a mol wt of ~110,000.[19,20] This band was intensified on exercise. In addition, considerable heterogeneity of the t-PA–

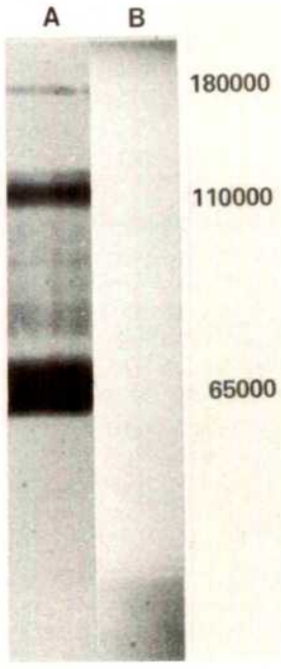


Fig 3. Zymography of euglobulin fraction of postexercise sample without immunoglobulin (track A) and with anti-tissue plasminogen activator (anti-t-PA) immunoglobulin (track B).

related activity at ~110,000 mol wt was observed in postexercise plasma, but this remains unexplained. It was not due to plasminogen-independent activity, which does not appear to increase on exercise or venous occlusion (Fig 2).

The band of ~180,000 mol wt in postexercise plasma and euglobulin was due to a complex between t-PA and C1 inhibitor (Fig 4). Purified t-PA, incubated with excess C1 inhibitor, formed a complex that retained some PA activity (track b), as has been shown previously for t-PA–PAI-1.[19,21] The complex of t-PA and C1 inhibitor had the same mobility with and without plasma and was indistinguishable from the 180,000 mol wt band seen in postexercise plasma. This band was removed from postexercise plasma by passage over a column of anti–C1-inhibitor immunoglobulin linked to Sepharose. On immunoblotting with antibodies to C1 inhibitor, postexercise plasma showed a very faint band of 180,000 mol wt in addition to a stronger band of ~120,000 mol wt, observed in both pre- and postexercise samples (data not shown).

Serial studies on individuals exercised to exhaustion. Serial studies in the subjects exercised on the bicycle ergometer allowed observation of the time scale of the development of these new bands of PA activity and correlation of their development with changes in the crude studies of plasma PA activity by the fibrin plate and euglobulin lysis time techniques.

Figure 5 (top) shows that the broad band of PA activity of ~60,000 mol wt started to appear in whole plasma at between 30 and 60 minutes, the time when PA activity became detectable in whole plasma applied to fibrin plates. It was fully developed at 60 minutes. This band remained detectable in the 95-minute specimen taken 15 minutes after complete exhaustion and cessation of all exercise, but had completely disappeared 4 hours after cessation of exercise. The t-PA–C1 inhibitor complex (180,000 mol wt) was also fully developed at 60 minutes, was weaker at 95 minutes—15 minutes after exercise ceased—and had disappeared completely 4 hours after cessation of exercise.

The activities of the euglobulin fractions of these plasmas are shown in Fig 5 (bottom). A series of changes occurred similar to that observed in plasma. The 110,000 band of t-PA–PAI-1 complex is clearly present in the euglobulin fraction; no activity was seen in the supernatant after euglobulin preparation. All the euglobulin PA was identified as t-PA (Fig 3). An additional band just above the 110,000 mol wt band was apparent in the 60- and 80-minute samples. The nature of this is unexplained, but it may be a complex of t-PA with another plasma inhibitor. No convincing evidence for a complex with α_2-antiplasmin or α_1-antitrypsin came from immunoblotting with antisera to these inhibitors.

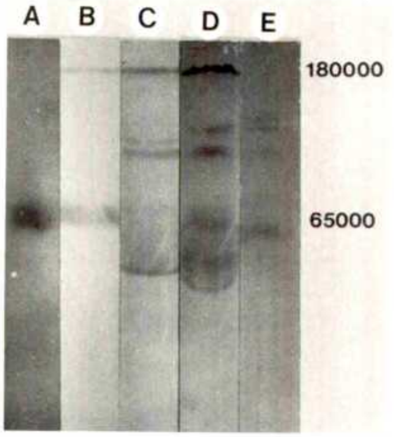
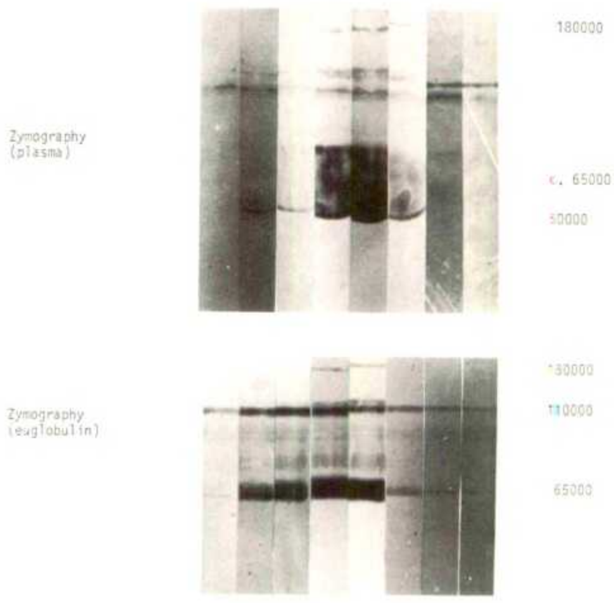


Fig 4. Zymography of (A) tissue plasminogen activator (t-PA) (5 mIU); (B) t-PA (5 mIU) plus C1 inhibitor (12 μg), preincubated for 30 minutes at 37°C; (C) t-PA plus C1 inhibitor as in (B) plus normal plasma; (D) postexercise plasma; and (E) postexercise plasma after passage over antibodies to C1 inhibitor–Sepharose.

Time (min)	0	15	30	60	80	95	320	470



Fibrin plate lysis (plasma; mm diam)	0	0	11	17	18	13	0	0
Fibrin plate lysis (euglobulin; mm diam)	13	16	20	23	23	21	14	14
ELT (min)	240	180	55	15	15	47	210	210

Fig 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography of plasma (top) and euglobulin fractions (bottom) before, during, and after exercise to exhaustion. Exercise commenced at time 0 and continued until exhaustion, which occurred in this subject at 80 minutes. Subject had a VO_{2max} of 62 ml/min/kg and averaged 72% of $VO^{2\ max}$ during this period of exercise.

Generation of plasmin during exercise. Intravascular generation of plasmin was examined using two-dimensional immunoelectrophoresis against antiserum to α_2-antiplasmin. Figure 6 shows that at the point of exhaustion a small but definite second peak was detected. Similar peaks were detected in three of the five subjects studied. This slow-moving peak represents plasmin-α_2–antiplasmin complex.[22] The complex was almost completely cleared 15 minutes after exercise ceased.

Patterns of PA after venous occlusion. Blood samples obtained with and without venous occlusion were analyzed by the same methods. The observed changes in fibrinolytic parameters (data not shown) were indistinguishable from

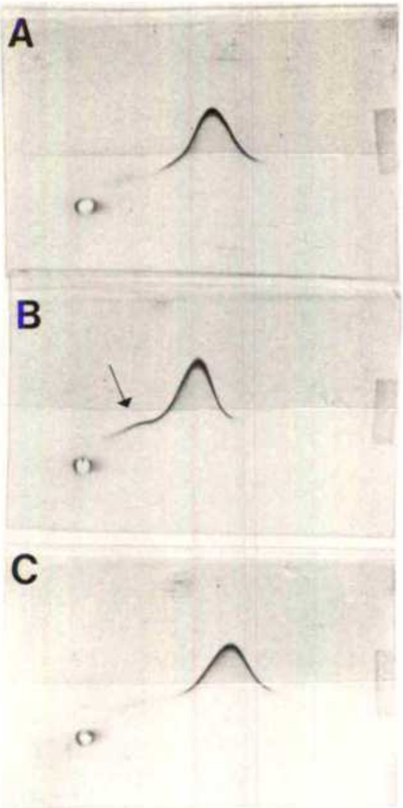


Fig 6. Two-dimensional immunoelectrophoresis of whole plasma against antiserum to α_2-antiplasmin (A) before exercise, (B) at point of exhaustion and (C) after 15-minute rest. Small peak of plasmin-α_2-antiplasmin complex is indicated by arrow.

those caused by strenuous exercise, except that no plasmin-α_2-antiplasmin complex was observed in any of the subjects studied.

DISCUSSION

This study examined the relative roles played by t-PA and u-PAs in the well-known increase in fibrinolytic activity following physical exercise and venous occlusion. The technique of SDS-PAGE followed by zymography allowed demonstration that this response is primarily due to t-PA–related material, which appears in several different mol wt forms, all neutralized by antiserum to purified t-PA.

The appearance in plasma samples of the broad band of t-PA–related material of ~60,000 mol wt in the exercise experiment coincided in time with the appearance of detectable PA activity in whole plasma when applied to fibrin plates. This band probably reflects free t-PA in the plasma, even though it migrates as a broader band of faster mobility than purified t-PA in the absence of plasma. Albumin considerably broadens the t-PA band and increases its mobility.[23] In experiments not illustrated here, we showed that the addition of purified t-PA to normal plasma results in its migration as a broad band of ~60,000 mol wt, indistinguishable from that seen in postexercise plasma samples in this study.

The t-PA–related band of ~180,000 mol wt was shown to be due to a complex with C1 inhibitor. A complex was formed in vitro with purified proteins, when C1 inhibitor was present in excess, and the complex retained some PA activity on zymography. Its mobility was unaffected by the presence of plasma proteins and its apparent mol wt was indistinguishable from the 180,000 mol wt band observed after exercise to exhaustion. The band was removed from postexercise plasma by passage over a column of antibodies to C1 inhibitor.

The 110,000 mol wt band in normal samples is a complex of t-PA with its major circulating inhibitor,[19,20] a platelet release product of ~48,000 mol wt,[21-24] which is also produced by endothelial cells[24-26] and is known as PAI-1. This band of complex is intensified during exercise, an increase that is most clearly observed on zymography of euglobulin fractions (Fig 5). A similar increase in this PA band in the euglobulin fraction after venous occlusion was reported.[27] The t-PA–PAI-1 complex clearly is precipitated in the euglobulin fraction and was not detected in the supernatant fraction. In contrast, free PAI-1 is distributed about equally between the euglobulin and soluble fractions (N.A. Booth and B. Bennett, unpublished observations). The nature of the weaker band of slightly slower mobility than the t-PA–PAI-1 complex, observed in this study, is uncertain.

Evidence has been presented by others for the presence of complexes of t-PA with α_2-antiplasmin, C1 inhibitor and α_1-antitrypsin in plasma.[20,28] In this study, no complexed forms of PA with α_2-antiplasmin or with α_1-antitrypsin could be demonstrated by immunoblotting; in each case, only a single band of the free inhibitor could be seen. Immunoblotting of normal and postexercise plasma with antiserum to C1 inhibitor showed only a very faint band of 180,000 mol wt in the postexercise sample, in addition to the band of ~120,000 mol wt seen in both samples. Thus, immunoblotting is a less sensitive technique than zymography for the detection of traces of this complex.

In contrast to t-PA, no striking changes in plasma u-PA activity were observed after exercise. The 50,000 mol wt band of u-PA seen in resting plasma was also present after exercise, possibly slightly intensified. The interpretation is complicated by the fact that u-PA and free t-PA in the postexercise plasma are poorly separated in this electrophoretic system, in which the presence of a high concentration of albumin causes the t-PA to spread[23] so that it migrates almost as rapidly as u-PA. Thus, only in the presence of antibodies to one of these PAs can the two types be distinguished (Fig 2). Because the techniques used in this study are not quantitative, no definite conclusion on changes in plasma u-PA concentration after exercise can be reached from these data. If there is a change, however, it is minor and all the detectable u-PA is free, uncomplexed to inhibitor. u-PA in patients with alcoholic cirrhosis is also detectable only in free form in plasma,[12] even though in vitro studies show the PA inhibitor in plasma to be capable of forming complexes with u-PA as well as t-PA.[19] This is probably because zymography detects only two-chain u-PA, and most u-PA in plasma is in the single-chain form.[29] Single-chain u-PA is stable in plasma[30] and is not susceptible to inhibition by PAI-1.[31]

Despite the considerable increase in total PA activity in the blood after exercise and venous occlusion and the presence of free t-PA, it is evident from these studies that very little plasmin was generated in most subjects during the observations. The method using two-dimensional immunoelectrophoresis against antiserum to α_2-antiplasmin is sensitive enough to detect quantities of plasmin associated with clinical hemorrhage[15,16,22] but remains a relatively crude instrument in a study such as this. It did detect the generation of significant amounts of plasmin-α_2–antiplasmin complex in three men exercised to exhaustion. Similarly, Collen and co-workers[32] noted a small increase in this complex, assayed by latex agglutination, in three of four subjects after repeated exhausting exercise. In another study, in which complex was assayed by a radioimmunoassay, less marked increases in complex were noted after exercise than after venous occlusion,[33] in contrast to our results. All these studies suggest that in some physiological as well as pathological situations sufficient plasminogen activator may be released to overcome inhibition of plasminogen activation and to result in generation of small quantities of plasmin in the circulating blood.

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Establishment and Phenotypic Characterization of Three New Human Myeloma Cell Lines (U-1957, U-1958, and U-1996)

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Three new human myeloma cell lines (U-1957, U-1958, and U-1996) have been established in vitro. The cell lines are Epstein-Barr virus (EBV) negative, monoclonal, and aneuploid and should thus represent malignant cell populations and not EBV-carrying non-neoplastic B lymphoblastoid cell lines. The myeloma origin of the cell lines is also suggested by their capacity for production of monoclonal complete immunoglobulin (Ig) molecules (U-1957 and U-1958) or IgG light chains (U-1996) of the same type as the myeloma protein in vivo. All the cell lines have morphological features of plasmablasts-plasma cells but appear to represent slightly different stages of B cell differentiation. Thus, the U-1958 has plasma cell morphology, expresses only PCA-1 and OKT-10 but no other B cell antigens, and secretes 1.5 μg/mL of IgG/10^6 cells/24 hours. The U-1957 has plasma cell morphology and expresses Fc receptors and the LB-1 antigen in addition to the PCA-1 and OKT-10 antigens. This line produces only minimal amounts of IgG, which appears not to be secreted. The U-1996, finally, is a κ light chain producer, has a plasmablast morphology, and expresses LB-1 in addition to the PCA-1 and OKT-10 antigens. All three cell lines are chromosomally heterogeneous and contain several markers with a 14q+ abnormality as a common characteristic abnormality. These new myeloma lines have been in continuous culture for ~3 years and are instrumental in studies of various aspects of the biology of human myeloma.

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NUMEROUS ATTEMPTS have been made during the last two decades to establish human myeloma cells as continuously growing cell lines in vitro. These attempts, however, have met with little success despite the increased efforts to grow human myelomas that have been made following the introduction of the method to produce immunoglobulin (Ig)-secreting mouse hybridomas. Very few authentic myeloma cell lines have been documented since the first report on a human myeloma cell line—the λ chain secreting RPMI 8226—was published in 1967.[1] Thus, only U-266,[2] L-363,[3] KMM-1,[4] Karpas 707,[5] Fr,[6] KMM 56,[7] LB-831 and LB-832,[8] OPM-1 and OPM-2,[9] and NCI-H929[10] appear to be cell lines where the myeloma nature has been proven beyond doubt. Other cell-putative myeloma cell lines have had a finite life span or have been found to be Epstein-Barr virus (EBV)-genome-carrying B lymphoblastoid cell lines.[11,12]

Two major factors seem to account for this low rate of success in establishing human myeloma cell lines. First, as judged from existing cell lines and from the behavior of myeloma biopsy cells in short-term culture, human myeloma cells are very strict in their nutritional requirements. They can be grown only in rich tissue culture media and almost exclusively when these have been supplemented by additional growth-promoting factors, usually derived from feeder cells such as fibroblasts.[13] Second, the frequency of spontaneously established EBV-carrying non-neoplastic, B lymphoblastoid cell lines is high in cultures of myeloma bone marrow explants.[14] B lymphoblastoid cell lines always have a selective advantage over the slow-growing myeloma cells. A potential myeloma cell line will therefore be lost once the outgrowth of the EBV-carrying cells begins.[2,15]

This article describes the establishment and characterization of three new human myeloma cell lines derived from the ascites and pleural effusion, respectively, of two patients with terminal myeloma. The phenotypic and cytogenetic examination reveal that these three lines are true myeloma cell lines.

MATERIALS AND METHODS

Clinical Data

U-1957 and U-1958 were derived from the pleural effusions obtained from a 60-year-old man with plasma cell leukemia. The patient had been admitted to the hospital 1 year earlier with an advanced myeloma, and therepy was immediately instituted (vincristine, melphalan, cyclophosphamide, doxorubicin, CCNU, prednisone). Initially, the patient's peripheral blood plasma cell count was 1.8×10^9/L. Myeloma cells also constituted 30% of the bone marrow cells, and several osteolytic lesions existed. A serum M component of IgGκ (7.5 g/L) was detected by electrophoresis. After an initial response to therapy, the disease progressed. Apart from additional osteolytic lesions, the liver and pancreas became heavily infiltrated by myeloma cells. The patient eventually died from liver and cardiac insufficiency.

The U-1996 cell line was derived from ascitic fluid obtained before chemotherapy was instituted. The patient was a 70-year-old woman who was hospitalized due to dyspnea and increasing abnormal circumference resulting from pleural effusion and ascites in which immature plasmablasts, most of which had typical morphological features, were suspended. In addition, the normocellular bone marrow was infiltrated diffusely with such cells (~20%). In the peripheral blood, a small number (1.0×10^9/L) of myeloma cells could also be detected. No osteolytic bone lesions could be demonstrated roentgenologically. Laboratory data showed decreased serum immunoglobulin levels (IgG and IgA) and κ light chain urea. Severe nephropathy (serum creatinine 7.5 mg/dL) with secondary hyperparathyroidism revealed an advanced stage of a primary extramedullary (?) myeloma with a secondary bone marrow infiltration and leukemic course. Chemotherapy with vincristine, cyclophosphamide, melphalan, and prednisolone was ineffective and was

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complicated by development of bleeding from multiple gastric ulcers. The patient died of pneumonia and uremia. Autopsy revealed a multiple pleural and intrapulmonal tumor spread. Intraabdominally, a huge tumor mass probably developing from the dorsal peritoneum overgrew most parts of the gut.

Culture Methods

Cells were harvested from the effusions by density-gradient centrifugation using Ficoll 400 (Pharmacia, Uppsala, Sweden). In an attempt to define the optimal growth conditions, cells were then incubated in Costar (Cambridge, MA) cluster plates in various media and sera combinations. The media tested were RPMI 1640, F-10, F-12, Eagles minimal essential medium (MEM), Waymouth, Iscove's, and MCDB 105. The following sera were screened for their capacity to support growth and survival of the myeloma cells: fetal calf serum (FCS) from GIBCO (Grand Island, NY), newborn calf serum (NCS) from the same company, pooled human AB serum, horse serum, and reindeer serum. The possible growth-promoting effect of conditioned medium was tested by incubating cells in cluster plates in RPMI 1640 supplemented by 10% FCS and 30% of conditioned medium, obtained from a panel of human cell lines including B, T, and myelomonocytic cell lines, fibroblasts, glial cells, and various sarcoma and cancer cell lines.

Maintenance of established cell lines. The lines were grown in 25- to 50-mL Ehrlenmeyer Flasks. For U-1957 and U-1958, the culture vessels contained a monolayer of human adult skin fibroblasts to provide necessary conditioning factors. U-1996 can be maintained without feeder cells. All lines are maintained in RPMI 1640 or F10 supplemented with 10% FCS and antibiotics (100 IU/mL of penicillin, 50 μg/mL of streptomycin). One third of the medium is changed twice a week.

Growth characteristics. The growth rate and the maximal cell density were determined from growth curves as described.[15]

Colony formation agarose. A cell suspension $\sim 5 \times 10^5$ cells/mL in 5 mL of F-10 supplemented by 10% NCS and 0.30% to 0.37% agarose was layered in 60-mm Petri dishes with or without a monolayer of fibroblasts. Finally, 2 mL of medium were gently dropped on top of the agarose layer. Cultures were incubated at 37 °C in a humidified 5% CO_2/95% O_2 atmosphere and were examined for colonies twice a week for 6 weeks.

Morphological Methods

Transmission electron microscopy (TEM). Cells were centrifuged at 400 g, and the pellet was gently resuspended in warm (37°C) 2% glutaraldehyde in 0.1 mol/L of Na-cacodylate buffer containing 0.1 mol/L of sucrose and fixed overnight at room temperature. The cells were postfixed in 2% osmium tetroxide in 0.15 mol/L Na-cacodylate buffer. Further preparation was performed as reported in detail elsewhere.[16] Sections were then studied in a JEOL 100 C microscope run at 60 to 80 kV.

Scanning electron microscopy (SEM). Cells were fixed and postfixed as described above. They were then allowed to sediment onto coverslips covered by poly-L-lysine.[17] Cells were then dehydrated and subjected to critical point drying[18] and coated by gold palladium as described elsewhere.[19] The specimens were examined in a JEOL microscope 100 C with scanning attachment run at 40 kV.

Cytochemistry. A selected panel of cytochemical stains were used to characterize the cell lines: α-naphthylacetate esterase,[20] naphthol AS-D chloroacetate esterase,[21] acid phosphatase,[22] and β-glucoronidase.[23] The fixation and staining were performed as detailed elsewhere.[20-23]

Surface markers. The presence of Fcγ- and sheep RBCs (SRBCs) receptors was investigated by techniques previously detailed.[24] A panel of monoclonal antibodies directed against cell source antigens expressed by B-cells at different stages of the differentiation pathway was used (Table 1). The antigen expression by the cell lines was examined by conventional indirect immunofluorescence using a fluorescein-labeled sheep anti-mouse antiserum purchased from the National Bacteriological Laboratory, Stockholm.

Table 1. Panel of Monoclonal Antibodies Used to Characterize U-1957, U-1958, and U-1996 Human Myeloma Cell Lines

Antibody	Nature of Antigen	Differentiation Associated Surface Expression	Reference
B1	p32	Pan-B	25
B2	p160	Immature-virgin-B	26
B4	p120	"Pre"-pre-B-pan-B	27
BB1	p37	B lymphoblast	28
HH-1	p95	Immature B-virgin B	29
MHM6	p45	B lymphoblast	30
LB-1	ND	B lymphoblast	28
PCA-1	ND	Plasma cell	31
PC-1	ND	Plasma cell	31
VIL-A1	p100	Pre-B(myeloid cells)	32
OKT10	p45	Thymocyte/plasma cell	33
anti-TAC	IL-2 receptor	T and B cells	34

ND, not determined.

Ig production. Cell-surface-bound Ig on living cells and cytoplasmic Ig in fixed cells were studied using indirect immunofluorescence. The panel of anti-Ig antisera included rabbit antisera specific for Fab fragments of IgG, IgM, and IgA (Dako, Copenhagen), for IgD (a gift from M. Greaves, London and purchased from Behringwerke AG, Marburg-Lahn, FRG), IgE (a gift from Dr Hans Bennich, Uppsala, Sweden) and κ and λ (Dako). The FITC-conjugated swine anti-rabbit antiserum used in the second layer was from Dakopatts (Copenhagen). Ig secreted to the medium was measured by a radioimmunoassay (RIA). Antibodies specific for human Ig heavy (m, d, g) and light (κ, λ), chains (Dako) were coupled to cyanobromide-activated methylcellulose beads (Avicel; Merck, Darmstadt, FRG) as a solid phase. In an inhibition RIA, supernatant Ig was then measured by competition with ^{125}I-labeled purified Ig chains.[35]

Western blotting. To verify the light chain production from the established cell lines further, a Western blot was made using cell lysates. As positive controls, U-266 was used for λ-light chain production, and a CLL patient was used for the κ light chain production, respectively. Cytoplasmic proteins from 5×10^6 cells of each cell line were separated by electrophoresis on a 15% polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane using an electroblot apparatus with buffer consisting of 25 mmol/L of Tris, 192 mmol/L glycine and 20% methanol (pH 8.3). The membrane was blocked with 5% wt/vol nonfat dry milk[36] in phosphate-buffered saline (PBS). The same reagent used for blocking was utilized as a carrier for primary antibody (rabbit anti-λ and anti-κ, Pharmacia, Sweden) and secondary antibody (sheep β-galactosidase–conjugated anti-rabbit IgG, Pharmacia). To remove unreacted reagents, the same blocking buffer containing 0.5% Tween 20 was used for washing. The blots were reacted with primary antibody for 4 hours, washed three times over 1 hour with the blocking buffer, reacted with the β-galactosidase–conjugated secondary antibody, washed as above and treated with the substrate containing 0.1 mg/mL of 6-bromo-2-naphthyl-β-D-galacto(pyrano)side, 0.5 mg/mL of Fast Garnet salt (Sigma, St Louis) and 1 mmol/L of $MgCl_2$ in PBS.

Table 2. Characteristics of Myeloma Cell Lines U-1957, U-1958, and U-1996

Characteristic	Cell Line		
	U-1957	U-1958	U-1996
Morphology			
Cell type	Plasmacell		Plasmacell
Cell shape	Round-ovoid, minimal variation		Round-ovoid, extensive variation
Cell surface	Slender villi and blebs		Slender villi and blebs
Nucleus	Finely granular chromatin; few nucleoli Asymmetric localization of nucleus		Marginated heterochromatin, most cells have one to two nucleoli; asymmetric localization of nucleus
Cytoplasm	Well-developed Golgi apparatus and rough ER Numerous mitochondria		Moderately well-developed Golgi apparatus and rough ER; numerous mitochondria
Growth			
Growth pattern	Single cells		Single cells
Dependence on feeder cells or conditioned medium	Yes		No
Attachment to feeder cells	Yes, slow		Yes, slow
Dependence on "rich" medium	Yes		Yes
Growth in agarose	No		No
Population doubling time (h)	48	60	48
Maximum cell density (cells/mL)	5×10^5	3×10^5	5×10^5
Cytochemical profile			
ANAE	+	+	+
NSADCAE	−	−	−
Acp II	+ + +	+ + +	+ + +
β-GLU	+ + +	+ +	+ + +
Cell surface markers			
B1	−	−	−
B2	−	−	−
B4	−	−	−
LB-1	+	−	+
BB-1	−	−	−
PCA-1	+ +	+ +	+
PC-1	−	−	−
OKT-10	+ +	+ +	+
MHM6	−	−	−
C2	−	−	−
Vil-A 1	−	−	−
Fc γ-receptor	+	n.t.	−
E receptor	−	−	−
Il-2 receptor	−	−	−
Immunoglobulin production			
Cytoplasm IgG	+	+ + +	−
IgM	−	−	−
IgA	−	−	−
IgE	−	−	−
κ	+	+	+
λ	−	−	−
RIA (ng/mL)/IgM	−	−	−
IgG	−	1500	−
Karyotype	Aneuploid; many markers including 6q+ and 14q+; near diploid		Aneuploid; multiple markers including 1p−, 6q+ and 14q+; polyploid (71-172)
EBNA	−	−	−

Abbreviations: ER, endoplasmic reticulum; IL-2, interleukin 2; Ig, immunoglobulin.

Cytogenetic analysis. Metaphases were collected from the cultures by addition of colchicine to a final concentration of 0.05 μg/mL 60 minutes before harvest, followed by addition of a hypotonic HCl solution (0.75 nmol/L) for 20 minutes. After centrifugation, the cells were fixed in alcohol/acetic acid (3:1) for at least 1 hour with two or three changes of the fixation fluid. Thereafter, chromosomes were isolated by dropping the suspension containing the fixed cells on cold and wet object slides. Metaphases were stained by Q-[37] and G-banding.[38] In some cases, R-[39] and C-banding[40] were used for better identification of marker chromosomes.

EB nuclear antigen (EBNA). EBNA staining was performed according to Reedman and Klein.[41] Reagents were kindly provided by Dr Georg Klein, Stockholm. Each test included one anti-EBNA–negative control serum and two positive sera. The Burkitt lymphoma line Raji was used as positive control cells. The U-698 lymphoma line[42,43] served as negative control cells.

RESULTS

Establishment of Cell Lines

The search for optimal culture conditions for the three biopsies led to the same conclusion, namely that the combination of a rich medium, ie, RPMI 1640 or F-10, FCS at a concentration of 20%, and the presence of feeder cells, was superior to any of the other tested nutrients. The presence of feeder fibroblasts or glia cells could be replaced by the addition of 20% to 30% of media harvested after 24-hour incubation of such cells.

The U-1957 and U-1958 have remained dependent on feeder cells, whereas U-1996 became feeder cell-independent within a few weeks after the explantation. An unwanted complication from the use of established human skin fibroblasts as feeder cells was that mycoplasma was transferred from those cells to the myeloma cell lines.

The myeloma cells were readily identified in the cultures by their typical growth as single cells forming a monolayer of relatively immobile, paving-stonelike cells. Other cell types identified during the first week of cultivation were some degenerating mesothelial cells and lymphoblasts. In no instance, however, was the outgrowth of typical clumps of EBV-carrying lymphoblastoid cells observed.

Morphology

The U-1957 and U-1958 cell lines derived from the same patient have essentially the same light-microscopical appearance (Table 2 and Fig 1A and B). The cells have typical plasmablast-plasmacell appearance. The morphological heterogeneity within these lines is minimal. Some bi-, tetra-, and even octaploid, nucleated large cells are found, however. The ultrastructural examination shows round-ovoid cells with multiple surface blebs and sparse slender villi (Fig 2A and B). The nucleus has fine granular chromatin and only little heterochromatin. Most cells have no nucleoli. The cytoplasm has typical plasmacell appearance with well-developed rough endoplasmic reticulum (ER) in ordered structures and a prominent Golgi apparatus (Fig 3A and B). The U-1996 also has the basic features of plasmablasts-plasmacells (Fig 1C), but the morphological heterogeneity is extensive. The surface ultrastructure is similar to that of the other two lines (Fig 2C). Ultrastructurally, the nuclei contain one to two nucleoli in most cells, and the chromatin is clumped and marginated (Fig 3C). The rough ER is less well developed than in the U-1957 and U-1958 cell lines as is the Golgi apparatus.

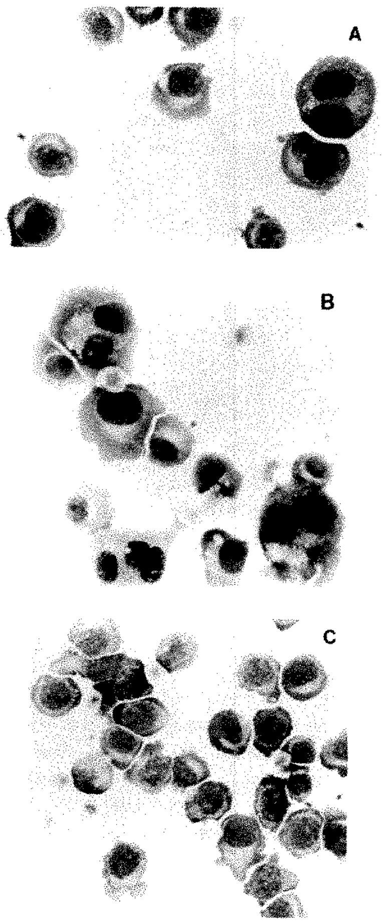

Fig 1. (A) U-1957, (B) U-1958, and (C) U-1996 cells. Cytocentrifuge preparations. Giemsa staining.

Growth Characteristics

The population doubling time varies between the cell lines, the U-1957 and U-1996 growing more quickly than the U-1958 (Table 2). The maximal cell density is only 3 to 5 × 10^5 cells/mL. None of the cell lines can be grown in agarose but can be cloned as single cells on feeder layers.

Cytochemical Profile

All lines had the same "myeloma cell-like" pattern (Table 2) expressing AcpII and β-glucoronidase.

Surface Markers

The pattern of antigen expression is shown in Table 2. Notable reactivities are as follows: All lines react with PCA-1 and OKT-10, and two of the lines (U-1957 and U-1996) react with LB-1. No line expresses the other B blast antigen (MHM6) or the pan-B antigens. The Fcγ receptor is weakly expressed. When present, each of the antigens is expressed on almost all cells but with varying intensity.

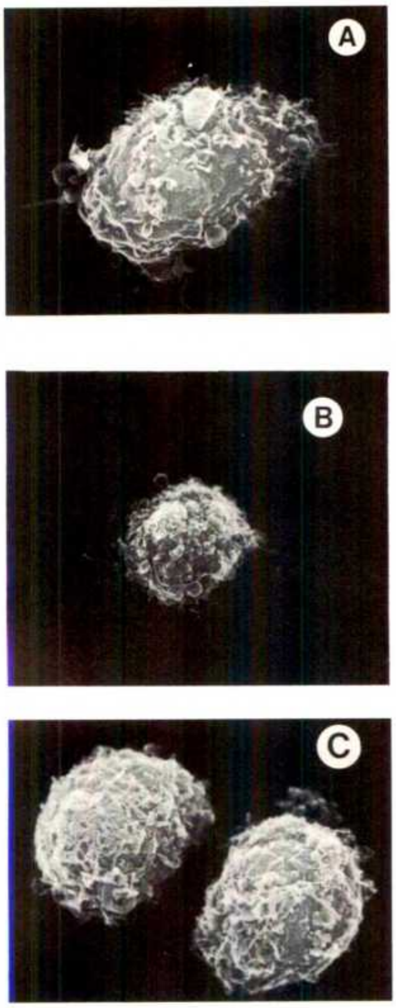

Fig 2. Scanning electron micrographs of: (A) U-1957 (original magnification ×8,000; current magnification ×3,600), (B) U-1958 (original magnification ×6,000; current magnification ×2,700), and (C) U-1996 (original magnification ×8,000; current magnification ×2,700).

Ig production

The U-1958 has strong cytoplasmic staining with the anti-IgG and is weakly positive with the anti-κ antibodies, whereas in U-1957 weak reactivity with the anti-IgG antiserum but no staining with the anti-light chain serum is found. The U-1996 cells stain weakly with anti-κ antiserum (Table 2).

The RIA analysis of the supernatant revealed that the U-1958 is a high producer of IgG. The rate of Ig secretion in the U-1958 cell line was $1.5\ \mu g/10^6$ cells/24 hours. No IgG was detected in the supernatant from U-1957 or U-1996.

Western Blotting

All cell lines gave immunoreactive bands only on incubation with the anti-κ light chain antiserum. The specificity of the reaction was proven by the incubation with the anti-λ light chain antiserum that gave negative bands as compared with the positive control for λ-light chain production (data not shown).

Cytogenetic Analysis

All cell lines were aneuploid when examined within a few weeks after being established as detailed.[44] In brief, the U-1957 and U-1958 were near diploid, but showed heterogeneity and many markers. The most frequently encountered markers were 1p−, 6q+, and 14q+. The latter marker appears to be produced by an interstitial duplication of a segment of 14q+. U-1996 was very heterogeneous both with respect to chromosome number (71-172) and markers. In this line, moreover, the 1p−, 6q+ and 14q+ markers were common abnormalities.

EBNA

All cell lines lacked the EBNA antigen.

DISCUSSION

The three cell lines described in this article are authentic myeloma cell lines by virtue of their morphology, pattern of Ig synthesis, chromosomal abnormalities, and EBNA negativity. They thus belong to the small group of true human myeloma cell lines reported.[1-10] The frequency of successful establishment of cell lines from a previous series of 100 ordinary myeloma bone marrow biopsies was zero regardless of the tissue culture technique used.[45] The explanation for this failure was, in 70%, the outgrowth of EBV-containing B lymphoblastoid cell lines that eventually overgrew the cultures. A second characteristic feature of myeloma cells in vitro, when obtained from patients with growth of the malignant clone restricted to the bone marrow, has been that they gradually die, although in many cultures small nonproliferative clusters of myeloma cells will remain viable for 3 to 4 months (terminal differentiation?). The reported permanent cell lines have, with one exception,[6] been derived from unusually widespread and aggressive myelomas, eg, pleural effusion and ascites or from cases with plasma cell leukemia. For the present study, we therefore selected only such patients for our attempts to establish new myeloma cell lines, and we have been successful insofar as five lines, including one lymphoblastoid, have been established from six patients.

The phenotypic characteristics of the new myeloma lines conform to a large extent with those of our U-266 and U-268 cell lines, derived from an IgEλ patient.[2,15,46] Taking the features of these cell lines and those of another newly established cell line, U-2030[47] together, one may conclude that human myeloma cells have certain unique in vitro biological characteristics. Thus, the morphology, both at the light microscopic and the ultrastructural levels, is quite typical, with plasmacell-plasmablast appearance; moreover, a fraction of the cells are binucleated and multinucleated cells. The medium requirement is usually stringent, as evidenced by the need for feeder cells or conditioned medium, at least at the time before and some time after the establishment. The growth pattern on feeder cells is also quite characteristic, as is the comparatively slow growth rate, low maximal cell density, and poor cloning ability.

The new myeloma cell lines appear to represent slightly different stages of B lymphoid differentiation as judged by the surface marker profile, the capacity for Ig secretion, and

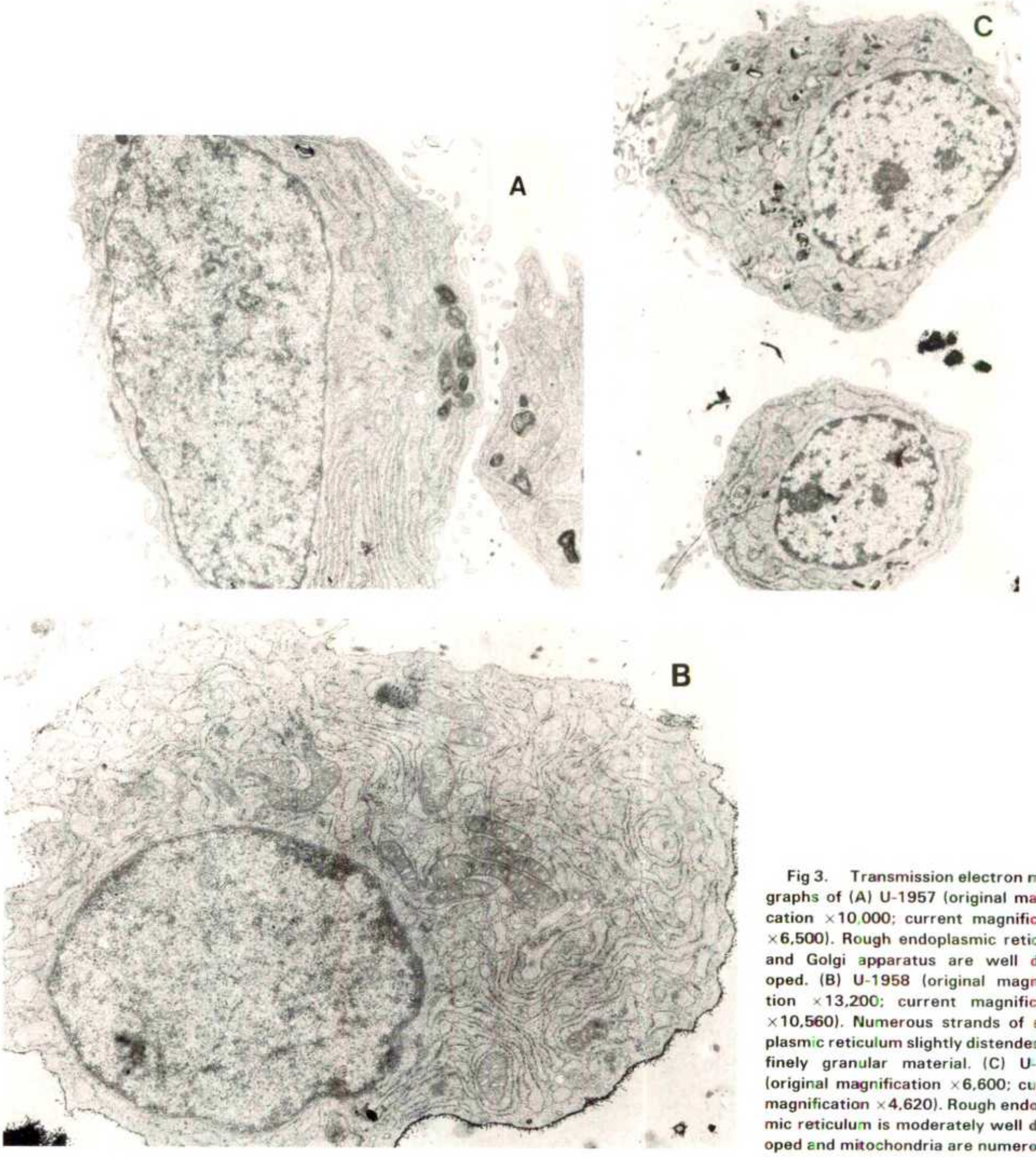

Fig 3. Transmission electron micrographs of (A) U-1957 (original magnification ×10,000; current magnification ×6,500). Rough endoplasmic reticulum and Golgi apparatus are well developed. (B) U-1958 (original magnification ×13,200; current magnification ×10,560). Numerous strands of endoplasmic reticulum slightly distended by a finely granular material. (C) U-1996 (original magnification ×6,600; current magnification ×4,620). Rough endoplasmic reticulum is moderately well developed and mitochondria are numerous.

the development of rough ER and Golgi apparatus. U-1996 is ultrastructurally the least differentiated of the three lines. Its relative immaturity appears to be reflected by the expression in this line of the LB-1 antigen, which is preferentially expressed at the lymphoblast stage.[48] The expression of plasmacell-associated antigens (PCA-1 and OKT-10) is low. The most important indicator of immaturity of U-1996, however, is the absence of capacity for production of complete Ig molecules. The U-1957 appears to be slightly more differentiated than the U-1996 but less differentiated than the U-1958. The former line has a well-developed ER and Golgi apparatus but expresses LB-1 and Fcγ-receptors and secretes no IgG that could be detected by RIA examination of spent supernatant. The U-1958, although derived from the same patient, represents an advanced stage of B cell maturation as demonstrated by the morphological, surface marker, and Ig synthesis criteria. The rate of Ig secretion appears to be equivalent to that of the U-266 cell line[15] but lower than that of the RPMI 8226.[49]

The cytogenetic analyses demonstrate extensive heterogeneity within the cell lines. Because U-1957 and U-1958 were examined shortly after the explantation in vitro, this probably reflects the fact that the tumor progression process was very advanced within the myeloma population in vivo.

The extensive chromosomal abnormalities and heterogeneity may explain not only the unusual growth behavior of the tumor in vivo but also the fact that cell lines could be successfully established in vitro.

The three cell lines have now been in continuous culture for >3 years. Throughout this period, the phenotypic characteristics have remained stable. This is surprising in view of the extensive chromosomal abnormalities found in all the lines, but reminiscent of the state of the U-266 cell line, which also is highly aneuploid but yet phenotypically very stable. Another paradox is that these aneuploid lines, like the U-266 during its first years of continuous cultivation, have not been able to mutagenize to become hypoxanthin-aminoptherine-thymidine (HAT)-sensitive. The chromosomal heterogeneity should reflect genetic instability, however, and our attempts to select sublines suitable for production of human hybridomas continue.

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Plasma Cyclic Nucleotide Levels in Acute Leukemia Patients

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To verify the clinical usefulness of extracellular cyclic nucleotide determination as a tumor marker, plasma cyclic AMP (cAMP) and cyclic GMP (cGMP) levels were measured in 70 normal subjects and 173 acute leukemia patients studied in different stages of their disease. Mean plasma cAMP levels were similar in leukemic and normal subjects, although in 48 patients in the active stage of the disease, first diagnosis, or relapse, the cAMP values were below the normal range, and most of these patients failed to respond to chemotherapy. Plasma cGMP levels were markedly elevated in untreated patients, normalized in all patients who attained complete remission, and increased promptly to pretreatment values in patients who relapsed, suggesting that their determination may be useful to monitor the patients' response to treatment.

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CYCLIC ADENOSINE 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) have complex and perhaps opposite regulatory influences on proliferative processes, but their role in the pathogenesis of malignant transformation is still unclear.[1-3] Alterations in the cyclic nucleotide metabolism have often been found in neoplastic tissue, however, although a consistent pattern of changes has not emerged.[4-10]

Recently, more attention has been paid to the alterations in extracellular cyclic nucleotide concentrations in human malignancies due to their potential clinical usefulness as tumor markers. Plasma and urine cAMP levels are generally unchanged in normocalcemic cancer patients, however, although increased or decreased cyclic nucleotide levels have also been reported.[9,11-21] On the other hand, increased cGMP levels and reduced cAMP/cGMP molar ratios in plasma and urine seem to be a more constant feature of cancer patients, with a relatively low frequency of false-negative values in the properly controlled studies.[9,11,12,14,15,17-21] Furthermore, our preliminary data and those of other researchers demonstrated that changes in cGMP levels and in cAMP/cGMP molar ratios correlated with the patients' response to treatment and with the occurrence of a relapse.[18-21]

We report here the results of a 3-year study performed to evaluate the clinical usefulness of plasma cAMP and cGMP level determinations in monitoring acute leukemia patients during the different stages of their disease.

MATERIALS AND METHODS

Plasma cyclic nucleotide levels were measured in 70 healthy volunteers, 30 males and 40 females, aged 18 to 78 years, (mean 34.3 years) and in 173 acute leukemia patients, 103 males and 70 females, aged 12 to 91 years (mean 37.9 years). The leukemia group consisted of 113 recently diagnosed and untreated patients, 54 with acute lymphoblastic leukemia (ALL) and 59 with acute nonlymphoblastic leukemia (ANLL), and of 60 treated patients, 28 with ALL and 32 with ANLL, who were in complete remission. Plasma cyclic nucleotide levels were subsequently reevaluated in 52 of the recently diagnosed patients, 26 with ALL and 26 with ANLL, after they had attained complete remission, and in 66 patients, 30 with ALL and 36 with ANLL, who relapsed. In addition, plasma cyclic nucleotide levels were monitored monthly during chemotherapy in 18 ALL and 22 ANLL patients who eventually relapsed and in 57 patients, 28 with ALL and 29 with ANLL, who remained in complete remission over the 12- to 36-month period of the study. Cyclic nucleotide levels were also measured in both plasma and CSF of eight patients, five with ALL and three with ANLL, with an isolated leukemic involvement of CNS. The control group consisted of six ALL and five ANLL patients in complete remission without any evidence of CNS leukemia, who had previously received prophylactic CNS therapy, and who underwent lumbar puncture as a part of the procedures to evaluate the possibility of stopping chemotherapy. All patients gave their informed consent to the lumbar puncture.

All patients had normal serum creatinine and blood calcium levels. Subjects were asked not to take any drug during the 2 days before sampling, even though in a preliminary study there was no evidence that the most common antileukemic drugs given the day before influenced plasma cyclic nucleotide levels. All subjects were on unrestricted activity and diet except for coffee, tea, alcohol, and smoking, which were not allowed on the day before sampling.

Venous blood samples were drawn between 8 and 9 AM, after an overnight fast and at least 1 hour's rest. CSF samples were obtained by lumbar puncture. The samples were added to prechilled tubes containing EDTA to give a final concentration of 5 mmol/L, immediately centrifuged at 4°C, and plasma and CSF were separated and stored at −80°C until assayed.

cAMP was measured by a protein binding assay and cGMP by radioimmunoassay (RIA) using commercially available kits (Cyclic AMP Assay Kit, Code TRK 432, and Cyclic GMP RIA Kit, Code 500; Radiochemical Centre, Amersham, England). Before being assayed for cyclic nucleotides, plasma and CSF were extracted with ethanol.[22] All samples were tested in triplicate using at least two different dilutions. The intra- and interassay coefficients of variation were $<10\%$ for both cAMP and cGMP.

Statistical significance of the data was evaluated by one-way analysis of variance using Duncan's and Kramer's tests for multiple comparisons.

RESULTS

Table 1 shows mean plasma cyclic nucleotide levels in the normal subjects and in the acute leukemia patients studied in different stages of their disease. There were no age- or

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Table 1. Plasma Cyclic Nucleotide Levels in Acute Leukemia Patients Studied in Different Stages of Their Disease

Diagnosis	Cases (n)	cAMP (pmol/mL)	cGMP (pmol/mL)	cAMP/cGMP
Normal	70	14.33 ± 0.389	4.11 ± 0.093	3.56 ± 0.108
ALL first diagnosis	54	12.63 ± 0.564	11.60 ± 1.622*	1.51 ± 0.118*
ALL complete remission	54	14.70 ± 0.405	3.69 ± 0.114†	4.12 ± 0.132†
ALL relapse	30	12.21 ± 0.821	8.68 ± 1.232‡	1.80 ± 0.140§
ANLL first diagnosis	59	14.12 ± 0.581	13.64 ± 1.970*	1.52 ± 0.095*
ANLL complete remission	58	14.59 ± 0.393	3.73 ± 0.086†	4.00 ± 0.129†
ANLL relapse	36	12.03 ± 0.621	9.96 ± 2.103§	1.76 ± 0.132§

Abbreviations: ALL, acute lymphoblastic leukemia; ANLL, acute nonlymphoblastic leukemia.

Data are expressed as mean ± SE.

*$P < .01$ *v* normal (Kramer's test).

†$P < .01$ *v* first diagnosis and NS *v* normal (Kramer's test).

‡$P < .05$ *v* complete remission and NS *v* first diagnosis (Kramer's test).

§$P < .01$ *v* complete remission and NS *v* first diagnosis (Kramer's test).

sex-related differences in the cyclic nucleotide values in any group of subjects. Leukemic patients showed mean plasma cAMP levels similar to those of normal subjects, independent of the stage of the disease. Plasma cAMP levels were below the normal range (9.70 to 29.35 pmol/mL), however, in 25 recently diagnosed patients, 13 with ALL and 12 with ANLL, and in 23 patients in relapse, 11 with ALL and 12 with ANLL. On the other hand, in untreated acute leukemia patients plasma cGMP levels were significantly higher, and cAMP/cGMP molar ratios were significantly lower than those of the normal subjects. Plasma cGMP levels were above the normal range (2.42 to 5.92 pmol/mL) in 45 of 54 ALL and 48 of 59 ANLL patients, and cAMP/cGMP molar ratios were below the normal range (2.21 to 7.04) in 48 of 54 ALL and 52 of 59 ANLL patients. As previously reported,[18,21] chemotherapy per se did not influence plasma cyclic nucleotide levels, but plasma cGMP levels and cAMP/cGMP ratios became normal in all patients who attained complete remission and remained in the normal range during all the remission period (Fig 1). In patients who relapsed, plasma cGMP levels increased and cAMP/cGMP ratios decreased to the pretreatment values (Table 1). Figure 2, which illustrates the plasma cyclic nucleotide pattern in 40 periodically monitored patients who relapsed under maintenance therapy, shows that most patients evidenced changes in cGMP levels and in cAMP/cGMP ratios before bone marrow blast invasion was observed. Table 2 shows the main hematological findings of these 40 patients during the 4 months before the relapse. There were no apparent correlations between these data and changes in cyclic nucleotide plasma levels, though platelet count was <100,000/μL in four ANLL patients 1 month before relapse.

In patients in the active stages of the disease, first diagnosis, or relapse, plasma cGMP levels and cAMP/cGMP ratios did not correlate with the ability to respond to treatment, remission duration, or survival time. Plasma cAMP levels were below the normal range in 18 of 57 recently diagnosed and 19 of 42 relapsing patients who failed to respond to chemotherapy, however, but only in 11 of the remaining 80 responsive patients ($\text{chi-square}_{(1)} = 12.583$; $P < .001$).

Patients in complete remission had CSF cyclic nucleotide levels ranging from 9.95 to 20.62 pmol/mL for cAMP and from 2.79 to 4.89 pmol/mL for cGMP. These values were in

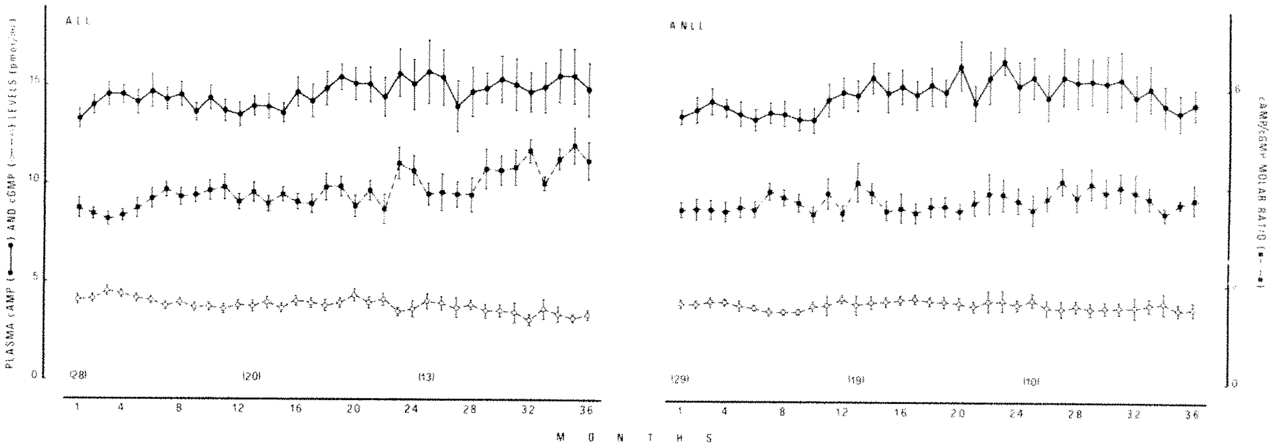

Fig 1. Plasma cyclic nucleotide levels in 57 treated acute leukemia patients who remained in complete remission. Of the patients with acute lymphoblastic leukemia (ALL), 13 were monitored for 3 years, 20 for 2 years, and 28 for 1 year; of the patients with acute nonlymphoblastic leukemia (ANLL), 10 were monitored for 3 years, 19 for 2 years, and 29 for 1 year. These numbers are shown in brackets. Data are expressed as mean ± SE.

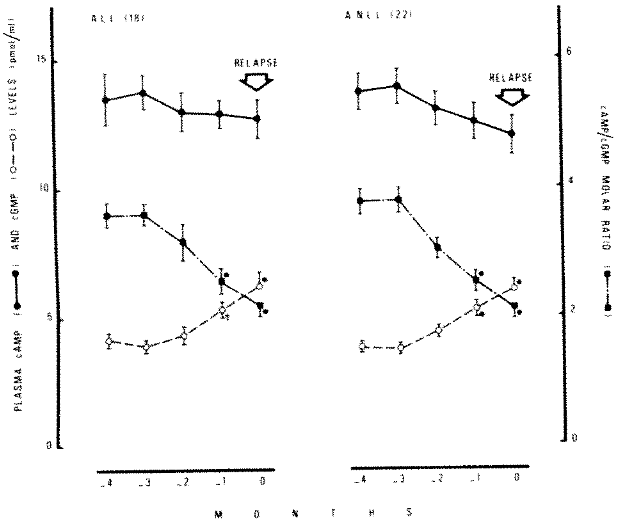

Fig 2. Plasma cyclic nucleotide levels in 18 acute lymphoblastic leukemia (ALL) and 22 acute nonlymphoblastic leukemia (ANLL) patients who relapsed under treatment. Reference time is that of relapse. Data are expressed as mean ± SE. *$P < .01$ *v* findings at −4 and −3 months (Duncan's test). †$P < .05$ *v* findings at −4 and −3 months (Duncan's test).

general agreement with those reported in the literature for subjects without evidence of CNS disease.[23,24] Patients with isolated CNS leukemia showed mean plasma and CSF cyclic nucleotide levels similar to those of the control subjects, with the exception of one ALL patient, with a symptomatic leukemic meningitis, who had CSF cGMP levels of 11.14 pmol/mL.

DISCUSSION

There were no significant differences in plasma cyclic nucleotide patterns between ALL and ANLL patients. Our results, however, indicate that in acute leukemias plasma cGMP levels and cAMP/cGMP molar ratios may be useful to monitor the patient's response to systemic chemotherapy and the occurrence of medullary relapse. On the other hand, plasma and CSF cyclic nucleotide levels were similar in patients with and without CNS leukemia, indicating that their determination does not have a role in the early diagnosis of CNS involvement.

Elevations in cGMP levels and reductions in cAMP/cGMP molar ratios in plasma and urine often occur in hematological malignancies[9,12,14,15,20,21] and in solid tumors,[11-15,17-19,21] although the frequency of normal values varies in the different studies. In our patients in the active stages of leukemia, first diagnosis, or relapse, the frequency of false-negative results was low, <15%, when either cGMP levels or cAMP/cGMP ratios were considered. The reduced cAMP/cGMP ratios generally reflected an increase in plasma cGMP levels; in ~20% of the patients, however, they were due to plasma cAMP levels below the normal range. In agreement with previously reported data indicating that decreased extracellular cAMP levels are not a frequent feature of normocalcemic cancer patients,[9,11-21] in this study only 48 patients had markedly reduced plasma cAMP levels; this finding, however, was an unfavorable prognostic indicator since most of these patients failed to respond to chemotherapy.

Because changes in extracellular cyclic nucleotide levels occur in different malignant diseases, they are not specific for leukemias and the mechanisms governing their development are still unclear. Extracellular cyclic nucleotide concentrations may reflect changes in their intracellular metabolism, since both adenylate cyclase-cAMP and guanylate cyclase-cGMP systems are unbalanced in human leukemic leukocytes.[8-10] Certainly, an enhanced egress of cGMP from cells[15] could account for the increased plasma cyclic nucleotide levels we found in the active stages of acute leukemias, though no direct evidence shows that cGMP is released from leukemic leukocytes rather than from normal tissues as a reaction to the malignant process. On the other hand, it seems unlikely that a reduction in cAMP levels in leukemic cells may in itself influence the plasma cyclic nucleotide concentrations, due to the many factors known to affect extracellular cAMP levels.

Studies in cancer-bearing animals have demonstrated that

Table 2. Main Hematological Data in 18 ALL and 22 ANLL Patients Who Relapsed Under Maintenance Therapy

Months Before Relapse	ALL							ANLL						
	Peripheral Blood			Bone Marrow				Peripheral Blood			Bone Marrow			
	Hb (g/dL)	WBC (× 10⁹/L)	Platelets (× 10⁹/L)	Blasts (%)	Cellularity* ↓	N	↑	Hb (g/dL)	WBC (× 10⁹/L)	Platelets (× 10⁹/L)	Blasts (%)	Cellularity* ↓	N	↑
−4	13.9	5.0	258	3.00	6	7	5	12.9	4.6	245	4.25	6	15	1
	(9.4–16.1)	(3.0–12.6)	(110–450)	(1.25–6.00)				(10.1–15.5)	(1.8–10.5)	(89–608)	(1.25–6.75)			
−3	13.9	4.5	259					12.8	4.9	258				
	(9.1–15.7)	(1.8–26.0)	(91–609)					(10.9–15.4)	(2.9–9.1)	(104–817)				
−2	13.8	4.7	240	2.75	6	8	4	13.0	4.6	275	4.75	8	9	5
	(10.5–16.6)	(3.1–15.8)	(105–731)	(0.25–7.25)				(9.8–15.5)	(1.7–9.4)	(95–658)	(2.00–11.50)			
−1	14.1	4.1	204					13.0	3.7	206				
	(9.1–16.8)	(2.2–15.0)	(125–429)					(10.7–16.0)	(1.8–10.9)	(16–552)				
0	13.5	5.4	134	38.0	6	5	7	12.4	3.2	152	26.0	10	7	5
	(10.3–16.9)	(1.2–21.7)	(6–350)	(10.0–67.0)				(6.5–15.5)	(0.8–17.0)	(12–408)	(10.0–89.0)			

Data are expressed as median and range for peripheral blood and bone marrow blasts.

*↓, Hypocellular, N, normocellular, ↑ hypercellular; the numbers under this heading refer to the number of marrows with the indicated level of cellularity.

extracellular cGMP levels correlate with tumor size.[25,26] A similar correlation could not be determined in our patients since the number of blasts in peripheral blood as well as the bone marrow richness are only indirect indicators of the leukemic burden. The finding that plasma cGMP levels and cAMP/cGMP molar ratios became normal in all patients who attained complete remission and changed precociously in patients who relapsed suggests, however, that these parameters behave as tumor markers. On the other hand, alterations in extracellular cyclic nucleotide levels have also been reported in some patients with benign tumors and with non-neoplastic diseases,[13,17,18,22,27] indicating that the finding of increased cGMP levels and/or reduced cAMP/cGMP ratios cannot be used in a previously undiagnosed subject as an unequivocal indication of the existence of a malignant process.

In conclusion, evidence shows that plasma cyclic nucleotide determination may be of clinical relevance as an additional parameter to monitor leukemic disease activity and may also suggest a more aggressive therapeutic regimen in recently diagnosed or relapsing patients with low plasma cAMP levels in view of their negative prognostic significance.

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Intermediate Lymphocytic Lymphoma: Immunophenotypic and Cytogenetic Findings

By Dennis D. Weisenburger, Warren G. Sanger, James O. Armitage, and David T. Purtilo

A detailed immunohistologic and cytogenetic analysis of 12 cases of intermediate lymphocytic lymphoma was performed. The characteristic immunophenotype of intermediate lymphocytic lymphoma was: surface IgM and IgD+, BA1+, B1+, BA2−, B2−, B4+, Leu 14+, Leu 1+, HLA-DR+, and common acute lymphocytic leukemia associated (CALLA) antigen negative. Clonal chromosome abnormalities were identified in ten cases, with structural or numerical abnormalities of chromosomes 11 or 12 in nine cases. Five cases had structural abnormalities involving the long arm of chromosome 11; three of these had translocations with chromosome 14 at band q32. Three cases had trisomy 12, and one case had a translocation involving the long arm of chromosome 12. The tenth case had a translocation involving the long arms of chromosomes 7 and 9. These characteristic immunophenotypic and cytogenetic findings suggest a close lineage relationship between intermediate lymphocytic lymphoma and small lymphocytic (well differentiated) lymphoma/chronic lymphocytic leukemia. Their differing clinical, cytologic, and architectural features suggest, however, that intermediate lymphocytic lymphoma should be considered a separate category of lymphocytic lymphoma in the International Working Formulation.

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INTERMEDIATE lymphocytic lymphoma is a histologically distinctive subtype of non-Hodgkin's lymphoma with cytologic features between those of small lymphocytic (well differentiated) lymphoma and small cleaved cell (poorly differentiated) lymphoma.[1,2] The architectural pattern of intermediate lymphoma is usually diffuse, although some cases have a follicular mantle-zone pattern (mantle-zone lymphoma).[3,4] Diffuse intermediate lymphoma has a clinical presentation and course similar to that of diffuse small cleaved cell lymphoma, with a median survival of only 30 to 31 months.[2,5] In contrast, mantle-zone lymphoma appears to be a relatively indolent form of intermediate lymphocytic lymphoma.[3,4] Immunologic studies of a small number of cases of intermediate lymphoma have demonstrated a B cell origin.[1,3,5-9] No cytogenetic studies of intermediate lymphoma have been reported, however. This study reports the immunophenotypic and cytogenetic findings in 12 cases of intermediate lymphocytic lymphoma.

MATERIALS AND METHODS

Lymph node biopsy specimens were obtained from 12 patients being studied and treated by the Lymphoma Study Group at the University of Nebraska Medical Center. All lymph nodes were processed for histologic, immunologic, and cytogenetic studies according to a standard protocol. Portions of each lymph node specimen were fixed in B5 and formalin for routine histologic processing, and hematoxylin and eosin-stained sections were prepared. A diagnosis of intermediate lymphocytic lymphoma was made on the basis of previously established histologic criteria.[2,3] The clinical records were reviewed, and the patients were staged according to the Ann Arbor classification.[10] The type of therapy, response to therapy, status at termination of the study, survival in months, and cause of death were recorded. The response to therapy was classified as a complete remission, a partial remission, or no response. A complete remission was defined as the total disappearance of all symptoms and clinically detectable disease. A partial remission was defined as a ≥50% disappearance of all known tumor and the appearance of no new lesions for at least 1 month. Survival was measured from the time of diagnosis.

Representative portions of fresh nodal tissue were prepared for frozen section immunohistochemical analysis. In brief, a 2.0-mm section of tissue was placed in optimum cutting temperature (OCT) embedding media (Miles Laboratories, Naperville, IL) and snap frozen at −150°C in isopentane quenched in liquid nitrogen. The tissue was then stored at −70°C until sectioned. Cryostat sections were air dried and fixed in cold acetone (4°C) for ten minutes. Immunohistochemical stains were performed with a three-stage immunoperoxidase technique[11] using unconjugated mouse antibody in the first stage, followed by biotin-conjugated goat anti-mouse $F(ab')_2$ antibody, and then by avidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA). Primary mouse antibodies to human immunoglobulins (Igs) G, A, M, D, κ and λ (Dako, Santa Barbara, CA), B cell antigens B1, B2, B4 (Coulter Immunology, Hialeah, FL), BA1, BA2 (Hybritech, San Diego), and Leu 14 (Becton Dickinson, Mountain View, CA), and T cell antigens OKT 4, 8, 11 (Coulter), and Leu 1 (Becton Dickinson) were used. Primary antibodies to common acute lymphocytic leukemia associated (CALLA) antigen (Coulter) and HLA-DR (Becton Dickinson) were also used. A case was considered positive for a specific cell surface antigen if most neoplastic cells stained clearly positive (weak or strong) for the antigen.

Flow cytometric analysis of peripheral blood mononuclear cells was also performed in four cases. The mononuclear cells were separated from heparinized peripheral blood by density centrifugation on Ficoll-Paque. The mononuclear cells were stained with unconjugated primary mouse antibodies to human κ and λ light chains, B1, BA2, Leu 1, HLA-DR and CALLA antigens. The cells were then washed with cold phosphate-buffered saline (PBS), incubated with FITC-conjugated goat anti-mouse $F(ab')_2$ antibody, washed, and quantitated with an Ortho 50 H cytofluorograph equipped with a Data General 2150 computer for data analysis.

Lymph node biopsy specimens involved by lymphoma were also received by the cytogenetics laboratory and processed within 1 hour after the biopsy procedure, or the specimens were similarly received, cultured, and processed through the fixation stage by a nearby

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hospital laboratory before shipment. The tissue was placed directly into RPMI 1640 media (GIBCO, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS) and gentamycin. The lymph node was mechanically minced and placed in culture at 37°C without mitogens for 24 hours. If sufficient tissue was available, a 48-hour culture was also performed. Two hours prior to the initiation of harvest, the cells were exposed to Colcemid (0.05 pg/mL). The cells were then resuspended in 0.074 mol potassium chloride for ten minutes and fixed with a methanol and glacial acidic acid solution (3:1). The fixation procedure was repeated three times, and slide preparations were made. The slides were aged for a minimum of 24 hours and G-banded with Wright's stain. All metaphase plates were microscopically analyzed, recorded, and photographed. Karyotypes were arranged according to the International System for Human Cytogenetic Nomenclature.[12] An abnormal clone was defined as either two or more cells with the same structural abnormality or the same extra chromosome, or three or more cells with the same missing chromosome. If mitoses could not be confidently analyzed, the mitotic cell was not included in the data.

RESULTS

Clinical features. The clinical features of the 12 patients are given in Table 1. The median age of the patients at the onset of disease was 60 years (range 42 to 80 years). The male to female ratio was 1:1. All patients had advanced disease. Splenomegaly and bone marrow involvement by lymphoma were commonly present (75% and 73%, respectively). Five patients (cases 2, 3, 5, 6, and 12) were considered leukemic at the time of initial diagnosis. The lymphoma cells in the peripheral blood and bone marrow were atypical prolymphocytes, as previously described.[2] The patients were treated with various chemotherapies. Four patients achieved a complete remission; two of these (cases 7 and 11) are presently alive and free of disease; the other two patients (cases 1 and 8) had prolonged disease-free survivals before the lymphoma recurred. Eight other patients are alive with or have died of lymphoma.

Histologic features. In 11 patients, the initial lymph node biopsy specimen showed effacement of the normal nodal architecture by a diffuse lymphoid infiltrate (diffuse intermediate lymphoma).[2] In one patient (case 5), the lymphoid cells infiltrated as wide mantles around benign-appearing germinal centers (mantle-zone lymphoma).[3] The infiltrating cells were atypical small lymphocytes with slightly irregular and indented nuclear contours (Fig 1A). There were too few cells with the uniformly round nuclei typical of small lymphocytic (well differentiated) lymphoma or the markedly angulated and cleaved nuclei of small cleaved cell (poorly differentiated) lymphoma to justify these diagnoses.

Immunologic features. Immunohistochemical stains revealed that all 12 cases of intermediate lymphocytic lymphoma were of B cell type (Table 2). Typically, intermediate lymphoma was positive for surface IgM and HLA-DR, and the antigens BA1, B4, Leu 14, and Leu 1 (Fig 1B). Seven cases expressed monoclonal κ light chains and five cases expressed monoclonal λ light chains. Variable positive staining was present for surface IgD (8 cases), IgG (2 cases), B1 (9 cases), and BA2 (2 cases). The neoplastic cells did not stain for surface IgA, B2 or CALLA antigens, or the T cell antigens OKT 4, 8, and 11. Moderate numbers of benign T cells were admixed, however, with a helper:suppressor cell ratio of 2 to 3:1.

Flow cytometric analysis of peripheral blood mononuclear cells was also performed in four cases (cases 2, 3, 5, and 10). A monoclonal B cell population was identified in three cases (cases 2, 3, and 5; Table 3); the other case (case 10) was normal. In the three monoclonal cases, the immunophenotypes of the circulating lymphoma cells were identical to those of the corresponding lymph node cells.

Cytogenetic features. Cytogenetic studies revealed a clonal chromosome abnormality in 10 of the 12 cases (Table 4). Nine cases had structural or numerical abnormalities of chromosomes 11 or 12. Five cases had structural abnormalities involving the long arm of chromosome 11; three of these had translocations involving bands q11-13 and chromosome 14 at band q32. Three cases had trisomy 12, and one case had

Table 1. Clinical Features of Intermediate Lymphocytic Lymphoma

Case	Age (yr)/Race/Sex	Splenomegaly	Absolute Lymphs/μL	Bone Marrow	Stage	Initial Therapy	Response	Survival (mo)	Cause of Death
1	54/W/M	+	2,035	−	IV B	COP	CR	D+ 113	Lymphoma
2	56/W/M	+	32,951	+	IV B	CL, P	NR	D+ 21	Lymphoma, bacterial pneumonia
3	75/W/M	+	1,925	+	IV B	CAP-BOP	ED	D+ 2	Lymphoma, herpes simplex pneumonia
4	63/W/F	+	1,122	−	III A	COPP	PR	A+ 87	NA
5	63/W/M	+	5,490	+	IV A	CLOP	NR	D+ 9	Lymphoma
6	80/W/F	−	10,586	+	IV A	CAP-BOP	PR	A+ 18	NA
7	53/W/F	+	930	+	IV A	COP, M	CR	A− 60	NA
8	59/W/F	+	5,850	+	IV A	CL	CR	A+ 204	NA
9	67/W/F	+	5,214	+	IV B	COP,BCNU	PR	A+ 32	NA
10	51/W/M	−	2,376	+	IV A	CLOP	PR	A+ 40	NA
11	60/W/F	−	1,032	ND	IV B	CHOP-B	CR	A− 27	NA
12	42/W/M	+	25,230	+	IV A	CL	PR	A+ 85	NA

Abbreviations: Lymphs, lymphocytes; W, white; +, present/positive; −, absent/negative; ND, not done; NA, not applicable; COP, cytoxan, vincristine, prednisone; CL, chlorambucil; P, prednisone; CAP-BOP, cytoxan, doxorubicin, procarbazine, bleomycin, vincristine, prednisone; COPP, cytoxan, vincristine, prednisone, procarbazine; CLOP, chlorambucil, vincristine, prednisone; M, methotrexate; BCNU, bischloroethyl nitrosourea; CHOP-B, cytoxan, doxorubicin, vincristine, prednisone, bleomycin; CR, complete remission; PR, partial remission; NR, no response; ED, early death; D+, dead with disease; A+, alive with disease; A−, alive without disease.

Table 2. Immunophenotypic Features of Intermediate Lymphocytic Lymphoma

	Surface Antigen-Positive Cases (n)														
Cases	IgM	IgD	IgG	IgA	κ	λ	BA1	B1	BA2	B2	B4	Leu 14	Leu 1	HLA-DR	CALLA
12	10	8	2	0	7	5	12	9	2	0	12	12	11	12	0

Abbreviation: CALLA, common acute lymphocytic leukemia associated.

a translocation involving the long arm of chromosome 12. The tenth case had a translocation involving the long arms of chromosomes 7 and 9. None of the cases had a translocation involving the long arms of chromosomes 14 and 18, as commonly occurs in the follicular center cell lymphomas.

DISCUSSION

Intermediate lymphocytic lymphoma is a common form of lymphocytic lymphoma, comprising 8.4% of all non-Hodgkin's lymphomas in the Nebraska Lymphoma Registry.[13] The clinical and pathologic features of the patients in this study are similar to those reported previously.[2-5] Patients with intermediate lymphoma are usually elderly and have advanced disease. Splenomegaly and bone marrow involvement by lymphoma are common. The clinical features of intermediate lymphoma are different from those of small lymphocytic (well differentiated) lymphoma, in which leukemia, usually with a high lymphocyte count, monoclonal gammopathy, autoimmune hemolytic anemia, and an indolent clinical course are common.[2-4,14-16] Although five (42%) of the patients in this study were leukemic at the time of initial diagnosis, the highest lymphocyte count was only 32,951/μL, and the cells were clearly atypical prolymphocytes rather than small lymphocytes. We believe, however, that an accurate diagnosis of intermediate lymphocytic lymphoma can only be made from well-fixed lymph node biopsy specimens using established histologic criteria.[2,3]

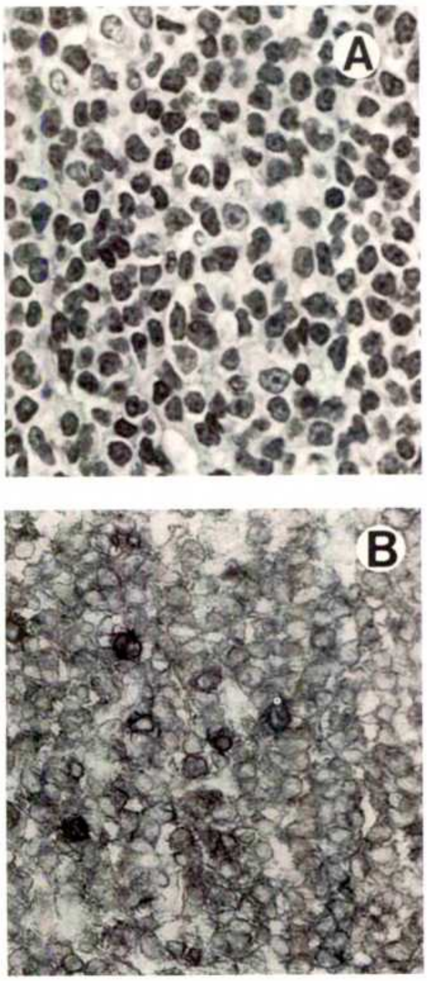

Fig 1. (A) Intermediate lymphocytic lymphoma. Most cells have slightly irregular and indented nuclear contours (hematoxylin and eosin stain, magnification ×100); (B) neoplastic cells stain slightly less intensely for Leu 1 antigen than do the few admixed normal T cells (immunoperoxidase stain, magnification ×100).

In this study, we determined the characteristic immunophenotypic and cytogenetic features of intermediate lymphocytic lymphoma. Using frozen section immunohistochemical stains, the cells of intermediate lymphoma typically bear surface IgM, usually with IgD, and the pan-B cell antigens B1, BA1, B4, and Leu 14. The cells also bear the T cell antigen Leu 1 and lack CALLA antigen. Our findings differ from those of Cossman and colleagues[7] who, using lymph node suspensions analyzed by flow cytometry, reported the expression of CALLA and BA2 antigen on most cases of intermediate lymphoma. However, Swerdlow and co-workers,[17] using a frozen section immunohistochemical technique similar to ours, reported that only 1 of 6 cases was clearly positive for BA2 antigen, and none were positive for CALLA antigen. However, Swerdlow and co-workers[17] did detect the weak expression of CALLA antigen on 1 of 4 cases studied by flow cytometry. These differing findings suggest that frozen section immunohistochemistry may fail to detect weakly expressed surface antigens.

Our findings indicate that the immunophenotype of intermediate lymphoma is identical to that of small lymphocytic (well differentiated) lymphoma/chronic lymphocytic leukemia.[1,18-20] The immunophenotype of intermediate lymphoma contrasts with that of the follicular center cell lymphomas, which usually bear surface IgG or IgA and CALLA antigen and lack IgD and Leu 1 antigen.[20,21] These findings suggest a closer lineage relationship of intermediate lymphoma with small lymphocytic lymphoma/chronic lymphocytic leukemia than with the follicular center cell lymphomas. Small lymphocytic lymphoma/chronic lymphocytic leukemia appears

Table 3. Immunophenotypic Features of Circulating Intermediate Lymphoma Cells

	Surface Antigen-Positive Cases (n)						
Cases	κ	λ	B1	BA2	Leu 1	HLA-DR	CALLA
3	3	0	3	1	3	3	0

Table 4. Cytogenetic Abnormalities in Intermediate Lymphocytic Lymphoma

Case	Age/Sex	Normal Cells (n)	Abnormal Cells (n)	Abnormal Clone
1	54/M	1	34	46,XY,del(11)(q14)
2	56/M	9	9	46,XY,t(11;14)(q13;q32)
3	75/M	0	4	46,XY,t(11;14),(q12;q32), del(13)(q22)
4	63/F	0	21	46,XX,t(1;?)(q32;?), t(6;13)(q23;q33), −11, +der(11),t(11;?)(q22;?), t(11;14)(q11;q32)
5	63/M	10	15	45,XY,t(2;9)(p14;p13), −4, +t(4;5)(q34;q12), t(8;11)(p12;q25), −9, del(13)(q13),t(13;?)(q34;?)
6	80/F	0	4	47,XX+12
7	53/F	5	10	49,XX, +12, +19, +21
8	59/F	0	2	48,XY, +12, +mar
9	67/F	0	4	46,XX, +r(4)(p16q31), t(4;6)(q31;q26), t(10;12)(q24;q22),t(12;?), (p13;?),del(14)(q21)
10	51/M	0	22	46,XY,t(7;9)(q11;q21)*
11	60/F	20	0	Normal
12	42/M	6	0	Normal

*Same clone found in the bone marrow.

to arise from an immature, bone marrow derived, virgin B cell.[22] The cell of intermediate lymphoma appears to correspond to a slightly more mature B cell that homes to and resides in primary lymphoid follicles and the mantle zones of secondary follicles.[3,4,21] In normal human ontogeny, by the seventeenth week of gestation, Leu 1-, IgM-, and IgD-positive B cells form the primary follicles of lymph nodes in close association with follicular dendritic cells.[23] Therefore, the intermediate lymphocyte appears to be the precursor cell of the normal germinal center.[21,23]

The cytogenetic abnormalities found in intermediate lymphoma are also similar to those reported for small lymphocytic (well differentiated) lymphoma/chronic lymphocytic leukemia.[24-32] Our studies revealed a clonal chromosome abnormality in 10 of the 12 cases. In the two cases without clonal abnormalities, the normal karyotypes were probably those of proliferating normal lymphoid cells within the tumor. Abnormalities of chromosome 11 are common in small lymphocytic lymphoma/chronic lymphocytic leukemia, the most common of these being a t(11;14) or deletion of 11q.[24-32] In small lymphocytic lymphoma/chronic lymphocytic leukemia, the break in chromosome 11 usually occurs in the region of band 11q13[26-28,31,32]; this was also true in four of our five cases of intermediate lymphoma. Band 11q13 appears to be a fragile site prone to frequent breaks in the normal lymphocytes of patients with small lymphocytic lymphoma.[25] The t(11;14) in small lymphocytic lymphoma/chronic lymphocytic leukemia and intermediate lymphoma causes juxtaposition of the cellular oncogene *bcl*-1 (11q13) with the immunoglobulin heavy chain gene (14q32).[33] Translocation- or deletion-mediated deregulation or activation of the *bcl*-1 oncogene may play an important role in neoplastic transformation and/or excessive cellular proliferation in the lymphocytic malignancies.[33] Trisomy 12 is also a common abnormality in small lymphocytic lymphoma/chronic lymphocytic leukemia.[24-32] Three of our cases of intermediate lymphoma also had trisomy 12, and one case had a translocation involving chromosome 12 at band q22. The cellular ki-*ras*-2 oncogene has been localized to chromosome 12 at bands p12 and q24.[34] Increased gene dosage or activation of the ki-*ras*-2 oncogene as a result of these abnormalities may result in an elevation of oncogene product, leading to neoplastic transformation and/or cellular proliferation.[34] None of our cases had the t(14;18) commonly seen in the follicular center cell lymphomas.[24-28,35] Thus, characteristic abnormalities involving chromosomes 11 and 12 have been demonstrated in intermediate lymphocytic lymphoma. These findings also suggest a close lineage relationship between intermediate lymphoma and small lymphocytic lymphoma/chronic lymphocytic leukemia.

In conclusion, the diffuse and mantle-zone variants of intermediate lymphocytic lymphoma are distinctive forms of non-Hodgkin's lymphoma with characteristic clinical, cytologic, architectural,[1-5] immunologic[1,4,5-9,17,20] and cytogenetic features. These findings suggest that intermediate lymphoma should be considered a separate category of lymphocytic lymphoma in the International Working Formulation.[36,37]

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Deficiency of Leukocyte Surface Glycoproteins Mo1, LFA-1, and Leu M5 in a Dog With Recurrent Bacterial Infections: An Animal Model

By Urs Giger, Laurence A. Boxer, Paul J. Simpson, Benedict R. Lucchesi, and Robert F. Todd III

A dog with severe recurrent bacterial infections, impaired pus formation, delayed wound healing, and severe persistent leukocytosis was the result of a mother–son mating. Assessment of leukocyte function revealed profound abnormalities in adherence-dependent activities including impaired granulocyte adhesion to glass/plastic surfaces or nylon wool, decreased granulocyte aggregation and chemotaxis, and diminished lymphocyte blastogenesis, but normal neutrophil oxidative activity, serum immunoglobulin, and complement levels. By immunofluorescence analysis, CD11b and CD18 monoclonal antibodies specific for the 155-kd α polypeptide of Mo1 (gp 155, 94) and the 94 kd β peptide common to Mo1, LFA-1 (gp 170, 94), and Leu M5 (p 150, 94) (surface molecules that promote leukocyte adhesion) failed to bind to unstimulated and A23187 calcium ionophore-stimulated granulocytes or mononuclear cells of the affected dog as compared with strong specific binding to canine control cells. The Mo1 glycoproteins were only barely detectable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of immunoprecipitates from lysates of ^{125}I surface-labeled neutrophils from the affected dog as compared with intense bands seen with canine control cell precipitates. We conclude that this dog has a severe leukocyte surface glycoprotein deficiency syndrome that is similar, if not identical, to that recently recognized in humans. Dogs with deficiency of leukocyte Mo1, LFA-1, and Leu M5 expression may represent a useful animal model to characterize further the molecular basis for an inherited disorder in leukocyte effector function.
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THREE STRUCTURALLY related adhesion-promoting cell surface glycoproteins, Mo1, LFA-1, and Leu M5 are expressed by human leukocytes, as detected by murine CD11a,b,c and CD18 (World Health Organization nomenclature) monoclonal antibodies (reviewed in refs. 1 and 2). They are $\alpha\beta$ heterodimers with an identical β subunit of 94 kd that is noncovalently associated with distinct α polypeptides of higher mol wt. The plasma membrane expression of these glycoproteins depends on the state of cellular activation and appears to be separately regulated despite sharing a common β-subunit.[3-5] Mo1 (gp 155, 94) has complement (C3bi) receptor type 3 (CR3) activity[6-8] and plays a role in myeloid cell adhesion phenomena, including substrate adherence, chemotaxis, and aggregation.[9-11] Lymphocyte function-associated antigen 1 (LFA-1; gp 170, 94) is expressed on phagocytes and lymphocytes and promotes lymphoid cell adhesion interactions that include lymphocyte proliferation and cytotoxic effector activity.[1,2,12-16] Leu M5 (p 150, 94), the most recently characterized phagocyte surface protein, may represent a lectinlike surface molecule, the physiological significance of which is as yet unclear.[2,17,18] Mo1 and/or LFA-1 glycoproteins (as detected by murine or rat monoclonal antibodies) have been identified in other species, including monkeys and other primates, mice, and dogs, indicating the conservation of these macromolecules during phylogeny.[19,20]

Recently, a combined Mo1, LFA-1, and Leu M5 leukocyte glycoprotein deficiency syndrome has been described in ∼50 children who demonstrate an increased susceptibility to infections.[4,5,21-32] This clinical syndrome is characterized by recurrent bacterial and fungal infections, progressive periodontitis, delayed umbilical cord separation, poor wound healing, and impaired inflammatory response. These patients have a persistent marked leukocytosis, with most of the cells being mature neutrophils, and profound abnormalities of adherence-dependent leukocyte functions. A moderate to severe deficiency of surface expression of leukocyte Mo1, LFA-1, and Leu M5 glycoproteins was noted in all cases, and a primary defect in the common β subunit of these glycoproteins is likely to underlie this syndrome.[5]

In this article, we report a leukocyte surface glycoprotein deficiency syndrome in the dog similar to that recognized in humans. The clinical features and the in vitro leukocyte functional abnormalities that characterize this disorder are described. CD11b and CD18 monoclonal antibodies reactive with canine Mo1, LFA-1, and Leu M5 facilitated our identification of the molecular basis of this animal's disease. In control dogs, the canine leukocyte surface glycoprotein complex was similar to that described in human and murine systems, and a severe deficiency of Mo1 and probably LFA-1 and Leu M5 glycoproteins was observed in the affected dog. This is the first report of an animal model of the human leukocyte Mo1, LFA-1, and Leu M5 deficiency syndrome.

MATERIALS AND METHODS

Case report. An 18-month-old female Irish setter cross-bred dog with a history of recurrent bacterial infections and persistent leukocytosis, first noted at the age of 2 months, was selected for study. The clinical presentations included deep skin wound infections, pododermatitis, superficial pyoderma, gingivitis, pneumonia, thrombophle-

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bitis, and ascending osteomyelitis of the right hind limb. The infection sites usually showed a localized cellulitis with impaired pus formation and were often associated with a local lymphadenopathy. Episodes of infection were commonly associated with severe pyrexia (40.6°C), anorexia, and weight loss. Poor wound healing after minor trauma was also noted.

Cultures of the skin infections usually grew *Staphylococcus intermedius*. *Escherichia coli* was cultured from an aspirate of a metatarsal bone and from skin affected by pododermatitis. *Pseudomonas aeroginosa* grew in the fluid aspirate of a subcutaneous cyst that contained many bacteria but few phagocytes when examined cytologically. Bacterial infections responded very slowly to appropriate systemic antibiotic therapy and local skin care. Because of a persistent osteomyelitis, fever and lameness recurred several times within a week after drug withdrawal. The dog was thereafter treated continuously with oral antibiotics. Fungal cultures and serum titers were negative, but *Trichuris vulpus* eggs were found on numerous fecal examinations. Antibody titers against distemper and parovirus infection appeared adequate following vaccinations with modified live virus vaccines.

Blood test results from the affected dog were collected for >1 year (Table 1) and revealed an extreme to moderate leukocytosis that persisted during apparently infection-free periods. The degree of the leukocytosis with WBC counts ≤208,000/μL was much greater than expected in normal dogs with similar bacterial infections. The persistent leukocytosis consisted predominantly of a mature neutrophilia, ranging from 21,000 to 188,000/μL, with a concomitant eosinophilia and variable lymphocytosis and monocytosis. Morphologic examination of leukocytes by light microscopy as well as routine leukocyte cytochemistry were normal except for occasional hypersegmented granulocytes and atypical lymphocytes. A bone marrow biopsy demonstrated marked myeloid hyperplasia. Episodes of severe leukocytosis were usually associated with moderate anemia of inflammatory disease.

A mild polyclonal gammopathy due to an increased α_2-protein fraction was also noted. Quantitative serum immunoglobulin concentrations and complement hemolytic activity (CH_{50}) levels were within normal limits, and direct Coombs' test and serum antinuclear antibody titer were negative.

The proband, an Irish setter cross, was one of five offspring of an accidental breeding between mother and son. The dam was a purebred Irish setter, and the sire was a cross between the proband's mother and a dog of unknown breed. One of the proband's littermates died during the first week of life; another male littermate had a history of recurrent bacterial infections and was euthanized. A female littermate and the parents of the proband did not exhibit any increased predisposition to infections and had normal leukocyte counts at least on one occasion. The last littermate was unavailable.

The studies were approved and in accordance with the school's guidelines for clinical research. An informed consent was received from the owner of the affected dog.

Table 1. Hematologic Data of Affected Dog

	Affected Dog*	Controls†
Hematocrit (%)	22-48	37-55
Leukocytes (× $10^3/\mu L$)	26-208	6-17
Segmented neutrophils	2.1-188	3-11.5
Nonsegmented neutrophils	0.1-2.4	0-.3
Lymphocytes	2.9-16.3	1-4.8
Monocytes	0.6-14.2	0.1-1.3
Eosinophils	1.4-15.6	0.1-0.8

*Data collected from 2 months to 1.5 years of age.

†Normal range for dogs, Veterinary Hospital, University of Pennsylvania, Philadelphia.

Source and preparation of leukocytes. Leukocytes from the affected dog with recurrent bacterial infection and leukocytosis were studied several times simultaneously with leukocytes from one or more control dogs. Data obtained from the affected animal were compared with results collected from 12 dogs of various breeds (including Irish setters) that were healthy, as determined by physical examinations and routine laboratory blood tests, and four dogs with typical infectious disease-associated leukocytosis. Venous blood was collected from a jugular vein in preservative-free heparin (10 IU/mL) or clot tubes and was processed within 4 hours. Polymorphonuclear leukocytes (PMNs) and mononuclear cells were prepared from heparinized venous blood by Ficoll-Hypaque centrifugation, gravity sedimentation with dextran and/or RBC lysis with hypotonic saline (0.2%) or 0.87% ammonium chloride at 4°C.[33]

Monoclonal antibodies. Murine monoclonal antibodies specific for 155-kd α subunit of Mo1 (CD11b IgG1 anti-Mo1 α [904]) and the 94 kd β subunit common to all three leukocyte surface glycoproteins Mo1, LFA-1 and Leu M5 (CD18 IgG2a IB4) were produced as previously described,[7,10,20] and were generously provided by Dr James Griffin (Dana-Farber Cancer Institute, Boston) and Dr Samuel Wright (The Rockefeller University, New York), respectively. Additional murine monoclonal reagents specific for the α subunits of Mo1 (CD11b antibodies LM2/1,[34] 44,[6] and 17[10]), LFA-1 (CD11a antibodies TS1/12,[12] TS1/22,[12] and CLB-LFA-1/2[14]), and Leu M5 (CD11c antibodies L29 and SHCL-3[17]), and the common β subunit (CD18 antibodies 60.3,[15] MHM23,[16] and TS1/18[12] from the CD11 and CD18 panels of the Third International Workshop and Conference on Human Leukocyte Markers were also used. Antibodies I-3 (9-49[35]), anti-Mo2 (CD14, 116[36]), and anti-PAM 1 (p181[37]) were used as isotype-identical negative control reagents.

Leukocyte function studies. The interactions of PMNs with various surfaces including cell adherence to plastic Petri dishes,[21] glass coverslips, and nylon fibers[38] were examined. In brief, PMNs (5 × 10^5) suspended in Dulbecco's phosphate-buffered saline (PBS) were allowed to settle in a 35-mm plastic Petri dish at 37°C. After a 45-minute incubation, the fluid was removed, the dish was gently rinsed with 2 mL of the above buffer, and adherent cells were fixed in 2% (vol/vol) glutaraldehyde in PBS for 1 hour at 4°C. The number and morphology of the adherent cells were evaluated by phase-contrast microscopy.[21] To study anchoring of cells to glass, freshly clotted blood was placed on a coverslip and incubated for 30 minutes as described for a neutrophil bacterial phagocytosis assay.[39] The coverslips were prepared and evaluated as described above for the Petri dishes. Finally, heparinized whole blood was filtered through 70, 110, 150, and 190 ± 10 mg nylon fiber packed in the bottom 25 mm of a Pasteur pipette, and the percentage of PMNs adherent was calculated as described by McGregor and colleagues.[38]

Random migration and chemotaxis under agarose were measured according to Nelson and colleagues[40] and described modifications,[41] using zymosan-activated serum as an attractant.

Aggregation of PMNs cells was performed as previously described.[9,42] In brief, canine neutrophils suspended in Hank's balanced salt solution (10^7/mL) were added to aggregation cuvettes and warmed to 37°C. Aggregation was induced by the addition of phorbol myristate acetate (PMA) (125 ng/mL) and was recorded as an increment in light transmission on an arbitrary scale, with use of a platelet aggregometer. Aggregation of normal canine PMNs after incubation with anti-Mo1 (904) or a nonreactive control monoclonal antibody for 10 minutes at room temperature was also measured.[9]

The oxidative metabolic burst of resting and stimulated PMNs was assessed by the product formation of a redox reaction and generation of reactive oxygen derivatives. The reduction of nitroblue tetrazolium (NBT) by PMNs in suspension was examined by the spectrophotometric method of Baehner and Nathan[43] (using 0.81-

μm latex spheres as stimulants), which in turn measures superoxide generation.[44] In flow cytometric studies, the oxidative product formation of the highly fluorescent 2′, 7′ dichlorofluorescein from the nonfluorescent substrate 2′, 7′ dichlorofluorescin was analyzed in single resting and PMA-stimulated cells according to Bass and co-workers.[45]

Lymphocyte proliferation response to phytohemagglutinin (2.5 or 5 μg/ml), concanavalin A (con A) (5 or 10 μg/mL), and pokeweed mitogen (PWM) (1 or 10 μg/mL) was measured in a microculture assay using canine heparinized whole blood.[46]

Immunofluorescence flow cytometry. Antigenic expression of Mo1, LFA-1, and Leu M5 leukocyte glycoproteins was investigated with mouse monoclonal antibodies against the α chain of Mo1 (CD11b, 904) and the common β chain (CD18, IB4) as previously described for human leukocytes.[3,4] In selected experiments, surface expression of neutrophil glycoproteins was assayed after stimulation by A23187 calcium ionophore (1 μmol/L) for 15 minutes at 37°C.[3,4] Indirect immunofluorescence flow cytometry was carried out by exposing PMNs to saturating concentrations of monoclonal antibodies for 30 minutes at 4°C and then, after washing, incubating them with a saturating concentration of fluorescein-conjugated goat antimouse immunoglobulin with added human IgG to block nonspecific binding mediated by Fc receptors. Immunofluorescence intensity of 5,000 leukocytes as a quantitative measure of relative antigen expression was analyzed on Coulter EPICS-C flow cytometer (Coulter Electronics, Hialeah, FL) using a logarithmic amplifier. To control for nonspecific binding of monoclonal anti-Mo1, LFA-1, or Leu M5 antibodies, parallel cell samples were exposed to isotype identical negative control monoclonal reagents.

Immunoadsorption and electrophoretic analysis of leukocyte antigens. Immunoadsorbed Mo1, LFA-1, and Leu M5 glycoproteins from lysates of ^{125}I-surface labeled PMNs and mononuclear cells were analyzed by SDS-PAGE (7.5% gels). In brief, canine neutrophils and mononuclear leukocytes were surface labeled with ^{125}I using the iodogen method.[47] These labeled cells were solubilized in 0.01 mol/L of Tris–0.15 mol/L of NaCl buffer, pH 7.8, containing 1% NP-40, phenylmethylsulfonyl fluoride (PMSF) 1 mmol/L, iodoacetamide 10 mmol/L, EDTA 10 mmol/L, and 100 μg (each) of leupeptin, pepstatin, antipain, and chymotrypsin. ^{125}I-labeled glycoproteins were isolated from NP-40 cell lysates by solid-phase immunoabsorption to immune complexes (murine monoclonal antibodies and affinity-purified F(ab′)$_2$ fragments of goat anti-mouse immunoglobulin) absorbed to polyvinyl plastic.[48] Washed immunoadsorbed antigen was solubilized in SDS sample buffer containing 2-mercaptoethanol and subjected to SDS-PAGE on 7.5% gels. Autoradiographs of dried slab gels were prepared, and the electrophoretic mobility of immunoadsorbed antigen was compared with that of protein standards.

RESULTS

Numerous in vitro leukocyte function studies were performed to characterize the leukocyte defect in the affected dog.

Adherence. Adhesion of the affected dog's PMN cells to the surface of glass coverslips, plastic Petri dishes, and nylon fibers was markedly impaired as compared with that of control canine PMNs. PMN adherence as a function of nylon fiber weight is shown in Fig 1. The percentage of the affected dog's PMNs adhering to the column was severely reduced at any nylon fiber weight between 70 and 190 mg. Complete adhesion of control dogs' PMN cells occurred at ≥150 mg nylon fiber, but was not achieved by PMNs from the affected dog. As indicated in Table 2, only few PMNs and mononuclear cells from the patient were adherent to the surface of glass coverslips or plastic Petri dishes. When examined by microscopy, the affected dog's cells did not flatten, but had a round, refractile appearance, and were easily dislodged by a gentle stream of buffer, whereas normal cells were firmly spread on substrate.

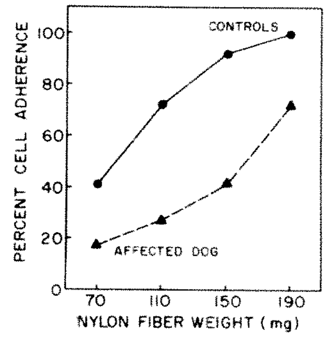

Fig 1. Nylon fiber adherence of polymorphonuclear cells from the affected and control dogs. Various weights of nylon fiber were packed in Pasteur pipettes. Each point represents the mean of two to five determinations. Affected dog (▲); control dogs (●).

Table 2. Leukocyte Function Studies of Affected Dog

Function	Affected Dog	Controls
PMNs adherence*		
(cells in ten fields)		
Plastic Petri dish	5 ± 4(3)†	78 ± 14(4)
Glass coverslip	3 ± 3(3)†	64 ± 11(4)
PMN cell migration‡		
(arbitrary migration units)		
Random migration	1 ± 1(3)	3 ± 1(3)
Chemotaxis (Zymosan)	2 ± 1(3)^2	8 ± 2(3)
NBT reduction§		
(OD)		
Resting	0.07 ± 0.03(3)	0.05 ± .01(5)
Phagocytizing	0.24 ± .07(3)	0.19 ± .04(5)
Flow cytometry of DCF oxidation‖		
(log green fluorescence)		
Unstimulated	19.2 ± 16.6(3)	8.3 ± 10.1(5)
PMA-stimulated	158 ± 21(3)	155 ± 22(5)
Lymphocyte blastogenesis¶		
(stimulation ratio)		
Con A (5/10 μg)	1.7/2.2	53/75
PHA (2.5/5 μg)	2.8/7.1	112/86
PWM (1/10 μg)	1/1	70/75

Abbreviations: PMNs, polymorphonuclear lymphocytes; NBT, nitroblue tetrazolium; DCF, 2′,7′ dichlorofluorescin; Con A, concanavalin A; PHA, phytohemagglutinin; PWM, pokeweed mitogen. Data are expressed as mean ± SD of (n), determination from affected dog or control dogs.

*Statistical difference between affected dog and controls, $P \leq .05$.

†Adherent cells in ten microscopic fields (× 100).

‡Migration measured by arbitrary microscopic units.

§Increased absorption at 515 nm caused by NBT reduction.

‖Hydrogen peroxide production by PMNs measured by DCF oxidation; data expressed as the mean log green fluorescence.

¶Representative experiment; stimulation ratio represents thymidine incorporation of stimulated over resting cells at two concentrations of stimulant.

Chemotaxis. The migration of the affected dog's PMNs as well as the migration in response to the chemotactic factor, zymosan-activated serum, was poor as compared with that of canine control cells, as shown in Table 2. Indeed, the attractant failed to stimulate chemotactic migration of PMNs from the affected dog significantly.

Aggregation. Canine control PMNs aggregated readily on stimulation with PMA (Fig 2A and B). In contrast, no significant aggregation of PMNs from the affected animal occurred in the presence of PMA (Fig 2C).

Oxidative burst reaction. No abnormalities were detected in the granulocyte "burst reaction" of the affected dog (Table 2). The reduction of NBT by PMNs in response to latex particle phagocytosis and the oxidation of 2',7' dichlorofluorescein by resting and PMA-stimulated cells from control and affected dogs was similar.

Lymphocyte proliferation. Mitogen-induced lymphocyte blastogenesis was markedly impaired in the affected dog as compared with controls (Table 2). Lymphocytes from the affected dog failed to proliferate in response to PWM, and only minimal responses were seen with con A and phytohemagglutinin (PHA).

Expression of Mo1, LFA-1, and Leu M5 canine leukocyte surface glycoproteins. With a panel of murine monoclonal antibodies (including the CD11 and CD18 panels of the Third International Workshop on Leukocyte Markers) specific for human Mo1, LFA-1, and Leu M5, normal and patient dog leukocytes were screened by immunofluorescence analysis for the expression of these glycoproteins. Among the reagents that cross-reacted with the PMNs of either normal dogs or control dogs with leukocytosis were CD11b antibodies 904 (shown in Fig 3), LM2/1, and 17 (each specific for epitopes on the α subunit of Mo1[10,34]), and CD18 antibodies IB4 (Fig 3), 60.3 MHM23, and TS1/18 (identifying epitopes on the β subunit polypeptide common to Mo1, LFA-1, and Leu M5[2,7,11,16]). Antibodies specific for the α subunits of LFA-1 (CD11a antibodies TS1/12, TS1/22, and CLB-LFA-1/2) or Leu M5 (CD11c antibodies L29 and SHCL-3) failed to bind to normal dog neutrophils (data not shown). In contrast to the reactivity of antibodies 904 and IB4 for control dog cells, no specific antibody binding to the neutrophils of the patient dog was observed (Fig 3). Because exposure of human (and dog) neutrophils to degranulating stimuli in vitro results in an up-modulation of surface Mo1 antigen from cytoplasmic granular pools,[3] we reexamined dog neutrophils after they were exposed to calcium ionophore A23187 to determine if patient dog neutrophils acquire detectable surface Mo1 expression from previously sequestered cytoplasmic stores. As shown in Fig 4, however, calcium ionophore A23187 treatment of patient dog neutrophils failed to stimulate the surface expression of Mo1.

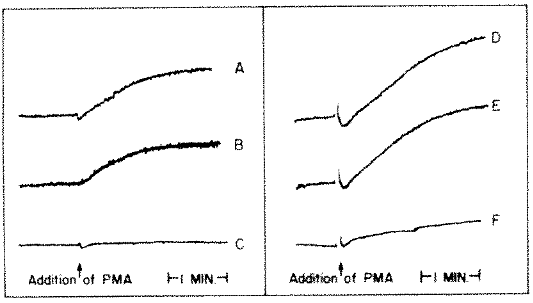

Fig 2. Aggregation of canine neutrophils from the affected dog and normal controls in response to phorbol myristate acetate (PMA); the effect of CD11b antibody 904 on aggregation of normal dog neutrophils. Leukoaggregation was recorded as the increment in light transmission (Δ T, *y* axis) over the time in minutes (*x* axis). Tracings A and B show the normal aggregation response of neutrophils from two control animals exposed to PMA. Neutrophils from the affected dog failed to aggregate in response to PMA (tracing C). Tracings D and E demonstrate the aggregation of normal canine neutrophils after pretreatment with buffer alone or with an IgG1 control murine monoclonal antibody (ascites, 1:50 dilution), respectively. Pretreatment of neutrophils with IgG1 monoclonal antibody 904 (specific for the α subunit of Mo1) attenuates the aggregation response (tracing F).

Consistent with the results of immunofluorescence analyses, very little Mo1 $\alpha\beta$ heterodimer was detectable in detergent lysates of ^{125}I-labeled neutrophils or mononuclear cells from the patient dog as compared with readily apparent Mo1 antigen isolated from the cells of a normal control individual. This is shown in Fig 5: In the normal control, the polypeptides characteristic of Mo1 (p180, 96) could be isolated from neutrophil lysates by immunoadsorbence to antibodies 904 (lane 4) or IB4 (lane 6), whereas only faint bands are visible in corresponding lanes 3 and 5 representing immunoadsorbed antigen isolated from the neutrophils of the patient. In lane 6 (antigen from normal dog neutrophils adsorbent to the anti-β polypeptide antibody), an additional band of 160 kd was observed, which, based on experience in the human system, probably represents the α polypeptide of Leu M5. Moreover, the existence of an LFA-1 α polypeptide distinct from Mo1 α is suggested by doublet appearance of the broad 180-kd band apparent in lane 6. Neither of these additional polypeptides was found in corresponding lane 5 (patient dog antigen). Likewise, no detectable immunoadsorbed β-associated antigen was detectable in the lysate of ^{125}I-labeled mononuclear cells from the patient (lane 10) relative to the three bands (180, 160, and 96 kd) isolated from the mononuclear cells of the normal control (lane 9). These results indicate that the leukocytes of control dogs express Mo1 and possibly LFA-1 and Leu M5 glycoproteins, but, in patient dog cells, the expression of these determinants is markedly deficient.

Mo1, which serves as an adhesion-promoting molecule of human neutrophils, performs a similar role in dog cells, as is suggested by the results shown in Fig 2: Anti-Mo1α antibody (antibody 904) attenuated neutrophil aggregation stimulated by PMA (tracing F) as compared with the normal response of neutrophils exposed to a negative control antibody (tracing E).

DISCUSSION

In this study, we identified a dog with recurrent severe bacterial infections whose leukocytes demonstrated an impairment in adhesion-related interactions and a deficiency in the expression of Mo1 and related adhesion-promoting glycoproteins. The abnormalities in leukocyte function

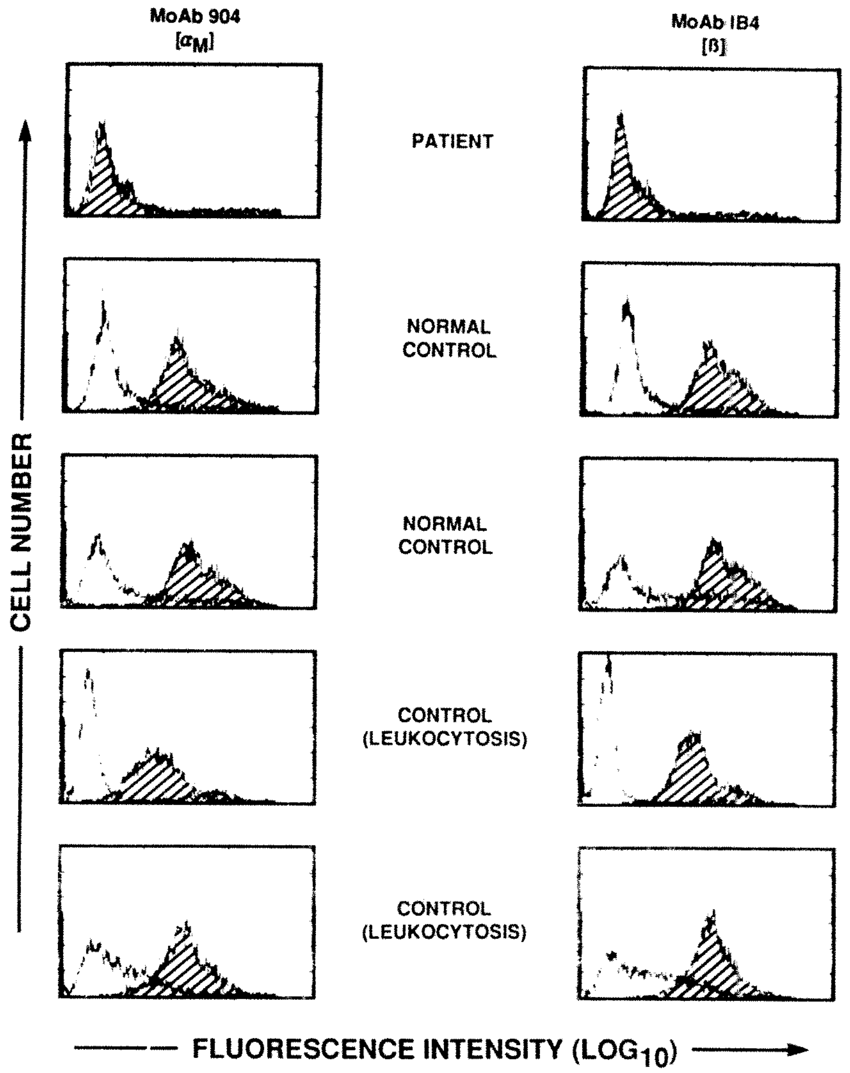

Fig 3. Surface expression of Mo1, LFA-1, and Leu M5 on neutrophils of the affected dog and control animals (either normal individuals or dogs with leukocytosis secondary to inflammatory conditions) as assessed by indirect immunofluorescence using murine monoclonal antibodies specific for the α subunit of Mo1 [CD11b MoAb 904 (αM) or for the subunit β common to Mo1, LFA-1 and Leu M5 (CD18 MoAb IB4) (β)]. The binding of antibodies 904 and IB4 (hatched histograms) relative to isotype-identical negative control antibodies (open histograms) to 5,000 neutrophils was measured by flow cytometry (cell number v fluorescence intensity).

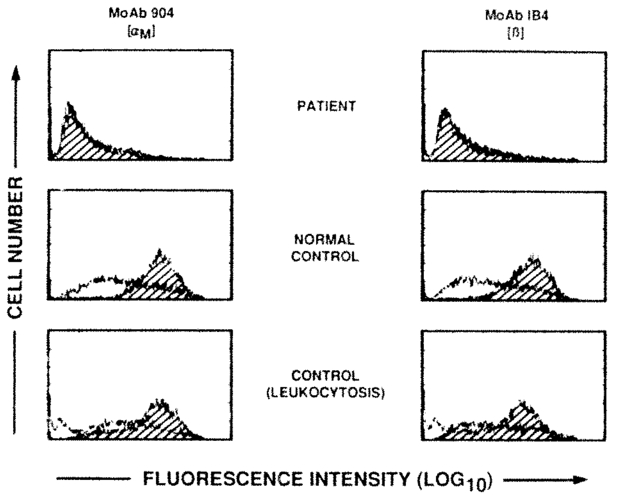

Fig 4. Surface expression of Mo1, LFA-1, and Leu M5 by neutrophils of the affected dog and control animals after exposure of cells to calcium ionophore A23187. As in Fig 3, hatched histograms represent the binding of antibodies 904 or IB4 relative to the binding of negative control antibodies (open histograms).

included significant decreases in neutrophil adherence to plastic, glass, and nylon wool, random migration and chemotaxis and aggregation in response to PMA, and a marked reduction in lymphocyte blastogenesis in response to mitogen stimulation (Table 2, Fig 1). Respiratory burst activity of the affected neutrophils was normal when latex particles or PMA was used as a stimulant (Table 2). A molecular basis for this impairment in adhesion-related leukocyte function was suggested by the deficient expression of leukocyte surface glycoproteins Mo1, LFA-1, and Leu M5, which, as shown in human and murine systems, contribute to leukocyte adhesion to substrates, chemotaxis, neutrophil aggregation, and to lymphocyte blastogenesis and cytotoxic function.[9-16,19] The inhibitory activity of anti-Mo1 monoclonal antibody 904 on canine neutrophil aggregation stimulated by PMA (Fig 2) indicates a similar role for Mo1 in the dog system. Deficient expression of Mo1 and related molecules was demonstrated by immunofluorescence analysis in which murine monoclonal antibodies specific for the α polypeptide of human Mo1 (CD11b) or the β polypeptide common to Mo1, LFA-1, and Leu M5 heterodimers (CD18) failed to bind to the neutrophils of the affected dog but demonstrated specific

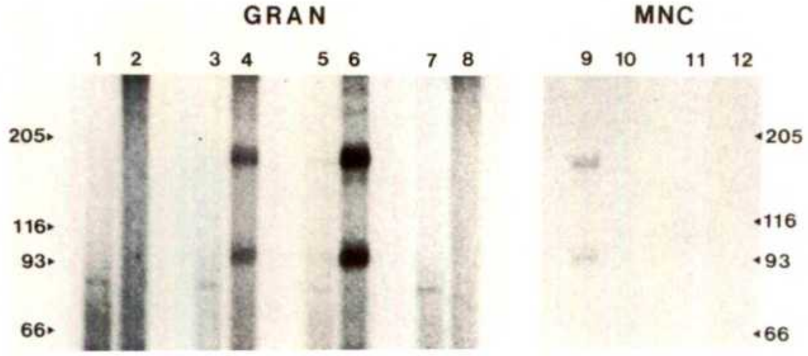

Fig 5. Immunoadsorption of Mo1, LFA-1, and Leu M5 from NP-40 lysates of ^{125}I-surface–labeled neutrophils (GRAN) or mononuclear cells (MNCs) of the affected dog (lanes 1, 3, 5, 7, 10, 12) or a normal control animal (lanes 2, 4, 6, 8, 9, 11). Equivalent amounts of ^{125}I lysate from the affected dog or the control individual were incubated on a solid-phase immunoadsorbent consisting of IgG1 anti-Mo1 antibody, 904 (lanes 3 and 4), IgG2a anti-β antibody, IB4 (lanes 5, 6, 9, and 10), an IgG1 negative control antibody (lanes 1 and 2), or an IgG2a negative control antibody (lanes 7, 8, 11, and 12). Immunoadsorbed antigen was solubilized in sodium dodecyl sulfate (SDS) sample buffer containing β-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis on 7.5% slab gels. Numbers represent electrophoretic mobility of protein standards. For comparison, human Mo1 antigen consists of two polypeptides of 170 and 96 kd when electrophoresed on 7.5% gels (not shown).

cross-reactivity with the cells of control dogs (including animals with leukocytosis secondary to inflammatory disorders). Stimulation of the affected dog's neutrophils by calcium ionophore A23187 under conditions that up-modulated Mo1 surface expression in normal animals failed to induce detectable Mo1 expression (Fig 4). Moreover, detergent lysates of ^{125}I-labeled leukocytes from the affected dog were markedly deficient in their content of immunoadsorbable Mo1 glycoprotein (p180, 96 on 7.5% polyacrylamide gels) relative to that isolated from a normal individual (Fig 5). α Polypeptides of LFA-1 and Leu M5 that were discernible (albeit weakly) after immunoadsorption to anti-β subunit antibody from lysates of normal canine cells were absent from that of the affected dog (Fig 5).

The disorder in leukocyte function in association with deficient expression of Mo1 and related glycoproteins exhibited by this dog is similar to a clinical syndrome recently described in >50 human patients with leukocytosis and recurrent bacterial infections.[4,5,21-32] In each case, the leukocytes of these patients demonstrated profound defects in neutrophil and monocyte adhesion to artificial and physiologic substrates, neutrophil and monocyte chemotaxis, neutrophil aggregation induced by a variety of inflammatory stimuli, as well as neutrophil and monocyte binding and phagocytosis of complement (C3bi-)-opsonized particles. Certain individuals exhibited impairments in delayed hypersensitivity reactions, including lymphocyte blastogenesis in response to soluble recall antigens and mitogens, and in cytotoxic T lymphocyte and natural killing. The leukocytes of these patients were either partially or totally deficient in the expression of Mo1, LFA-1, and Leu M5 glycoproteins, which, based on recent biosynthetic information,[49] may be the result of biosynthesis of a defective 94-kd β polypeptide common to all three glycoproteins.

Reports demonstrating the inhibitory effect of glycoprotein-specific monoclonal antibodies on leukocyte function indicate that Mo1 (expressed by monocytes and neutrophils) is the surface receptor for C3bi (CR3)[6-8] and also serves as an adhesion-promoting molecule of neutrophils and monocytes,[9-11] whereas LFA-1 (expressed by all leukocytes) facilitates adhesive interactions involving lymphocytes and natural killer (NK) cells[12-16] (the functional significance of Leu M5 has not been determined). Therefore, impairments in neutrophil and monocyte function (CR3 activity and adhesive interactions) exhibited by patient cells has been attributed to a deficiency in Mo1, whereas defects in lymphocyte and NK cell activity (blastogenic responses of lymphocytes; lymphocyte and NK cell cytotoxicity) are a consequence of a deficiency in LFA-1. By analogy, it is likely that the leukocyte functional abnormalities exhibited by the affected dog relate to the deficiencies in the expression of Mo1 and LFA-1.

Based on whether the deficiency in expression of Mo1, LFA-1, and Leu M5 is partial (2.5% to 31% of the glycoprotein expression of normal leukocytes) or complete (<0.3% of normal Mo1 expression even after calcium ionophore stimulation), two forms of the disorder have been identified in humans, with good correlation between the level of glycoprotein expression and the degree of leukocyte functional impairment and the severity of the clinical syndrome.[27] By the same criteria, the animal we studied would be classified as having a severe form of the disease with no Mo1 α polypeptide or common β peptide expression evident by immunofluorescence analysis and barely detectable amounts of Mo1 immunoadsorbed from cellular lysates.

Moreover, the clinical manifestations observed in this dog are unquestionably related to the profound adhesion dysfunctions of phagocytes in this disorder and are very similar to the clinical signs described in human patients with severe Mo1, LFA-1, and Leu M5 deficiency syndrome.[4,5,21-32] Bacterial infections of body surfaces such as skin and gingiva as well as osteomyelitis have previously been observed in human patients with this and other disorders that predispose to bacterial infections. Impaired pus formation and delayed wound healing appear to be more unique features of an abnormal inflammatory response in this disorder.

Of related interest are a series of reports by Renshaw and collaborators[50-52] who described a neutrophil function defect in a male Irish setter dog and some of its offspring. Dogs with this so-called "canine granulocytopathy syndrome" had a history of omphalophlebitis after birth and subsequent episodes of recurrent bacterial infections including pyoderma, pododermatitis, lymphadenopathy, gingivitis, and osteomyelitis. These animals had a persistent, marked leukocytosis, with most cells being mature neutrophils. A neutrophil defect in bactericidal activity due to a reduced glucose oxidation by the hexose monophosphate shunt was suspected, although these abnormal neutrophils showed increased capacity to reduce NBT dye. Unfortunately, most of these dogs died at a few months of age, and apparently none are available for further studies. Because the clinical features and routine laboratory test results appear to be identical with the disorder described here in an inbred Irish setter backcross, however, the previously reported "canine granulocytopathy syndrome" may indeed be caused by a leukocyte Mo1, LFA-1, and Leu M5 deficiency.

The study of family members of human patients with Mo1, LFA-1, and Leu M5 deficiency suggests either an autosomal recessive[4,5] or x-linked recessive[21] mode of inheritance. The parents of affected offspring are clinically normal and generally exhibit normal leukocyte function. Certain parents and clinically normal siblings, however, express intermediate quantities of leukocyte surface glycoproteins (best detected after maximal in vitro stimulation of leukocytes), suggesting a heterozygous state.[4,5]

The family history of the affected Irish setter mixed-breed dog suggests an autosomal recessive mode of inheritance, indicated because (a) the affected dog was a product of an Irish setter mother and her son (the father's father was of an unknown other breed), and (b) the parents and one female littermate were not afflicted by recurrent bacterial infections and had normal leukocyte counts, whereas another male littermate was reported to have a history of frequent bacterial infections. Preliminary leukocyte function tests and monoclonal antibody studies of mother and female littermate support an autosomal recessive inheritance with intermediate expression of leukocyte surface glycoproteins (data not shown). In addition, limited breeding studies of Irish setters with the previously described "canine granulocytopathy syndrome" also suggested an autosomal recessive mode of inheritance.[52]

In conclusion, the comparative evaluations of the clinical features, the functional leukocyte abnormalities, and the molecular defect between the affected dog and the previously described human patients with Mo1, LFA-1, and Leu M5 deficiency suggest that the patient has a leukocyte surface glycoprotein deficiency syndrome very similar if not identical to that recognized in humans. This is the first animal homologue of the leukocyte surface glycoprotein Mo1, LFA-1, and Leu M5 deficiency. We are presently trying to establish an animal colony. The availability of an animal model for this disorder should prove useful in further characterizing the molecular basis and pathophysiology of deficient leukocyte glycoprotein expression and in developing new therapeutic approaches. In particular, functional aspects of these adhesion-promoting leukocyte glycoproteins and lack of expression could be studied in vivo, and gene replacement therapy could be developed in a large animal species with this defect.

ACKNOWLEDGMENT

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Pluronic F-68 Reduces the Endothelial Adherence and Improves the Rheology of Liganded Sickle Erythrocytes

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A perfluorochemical blood substitute emulsified with Pluronic F-68 has been shown to improve the filterability of deoxygenated sickle red cells. We questioned whether some of the effect was independent of oxygen loading and studied the influence of Fluosol DA (Green Cross, Osaka, Japan) and Pluronic on the rheology and adhesion of sickle red cells saturated with oxygen and carbon monoxide. A 5-vol/vol% concentration of Fluosol or equivalent concentration of Pluronic was equally effective at improving the filtration of washed sickle cells through 5-μm-diameter pores at wall shear stresses approximating 1,000 dyne/cm². The same concentration of Pluronic reduced the extensional static rigidity of irreversibly sickled cells (ISC) by 25% and also abolished the adherence of gravity-sedimented sickle cells to endothelial monolayers in the presence of saline or plasma. The inhibition of adherence was not reversible by washing and was accomplished with equal ease by isolated treatment of sickle cells or endothelium. Pluronic had no effect on the rheology or adhesion of normal adult red cells. Neither Fluosol nor Pluronic changed sickle or normal cell shape, mean cell volume, mean cell density, or cell density distribution. A lubricating effect of Pluronic on cell surfaces could explain all of the rheological observations and offers another direction of inquiry in the search for therapy for sickle cell disease.
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RHEOLOGICAL ABNORMALITIES are apparent in oxygenated blood samples from patients with sickle cell anemia. Washed, oxygenated sickle red cells are less deformable than normal erythrocytes as evidenced by viscometry, filtration, ektacytometry, and micropipette aspiration studies.[1-6] Sickle red cells retaining an elongated, distorted shape under conditions of oxygenation are commonly termed irreversible sickled cells (ISC) and tend to be the least deformable of the sickle cells.[1,6] ISC are dehydrated, dense cells that have undergone permanent alterations of membrane protein and lipid organization.[7-9] Small amounts of intracellular sickle hemoglobin polymer persist in dense sickle cells after conventional oxygenation procedures, and exposure of oxygenated sickle cells to a higher-avidity hemoglobin ligand, carbon monoxide (CO), decreases the number of apparent ISC by approximately half.[10,11]

Sickle red cells also demonstrate increased adherence to each other and to cultured vascular endothelium.[12-15] The number of oxygenated sickle red cells adhering to endothelial monolayers correlates with the general clinical severity among sickle cell syndromes[13,16] and with the severity of vasoocclusive events in sickle cell anemia.[17,18] A greater number of washed, oxygenated sickle cells from the denser cell fraction adhere to endothelial monolayers than from the lighter sickle cell fractions.[14] Micropipette detachment studies suggest that the most distorted and least deformable sickle cells are less adherent to endothelial cells than intermediate forms,[15] perhaps due to a reduced ability to form multiple surface contacts.

Perfluorochemicals are chemically inert organic products of fluorination synthesis that dissolve large amounts of oxygen and are under active study as blood substitutes.[19-21] Fluosol 43, a perfluorotributylamine product emulsified with the nonionic detergent Pluronic F-68, improved the viscosity and filterability of deoxygenated sickle cells, presumably by improving oxygenation.[22] Curiously, deoxygenated sickle cells admixed with oxygenated Fluosol 43 were even more filterable than sickle cells oxygenated under ambient conditions.

We questioned whether the perfluorochemical emulsion exerted effects on red cell rheology independent of the improvement of oxygen loading. We therefore studied the influence of Fluosol DA, a new perfluorodecalin-perfluorotripropylamine product, and its emulsifier, Pluronic F-68, on the rheology of washed sickle and normal red cells saturated with oxygen and CO. Several aspects of red cell mechanical behavior were evaluated including filterability, cell shape, size, micropipette aspiration, and adherence to endothelial monolayers. Pluronic F-68 improved the filterability of sickle cells and micropipette aspiration of ISC and abolished sickle cell adherence to endothelium without altering cell shape or size. The studies are most consistent with a lubricating effect of Pluronic F-68 on the cell surface and consequent reduction of cell stickiness.

MATERIALS AND METHODS

Subjects. Blood samples were obtained from healthy adult volunteers and adult and pediatric patients with homozygous sickle cell anemia in accordance with the Committee on the Use of Human Subjects in Research of the University of Minnesota. The diagnosis of sickle cell anemia was verified by isoelectric focusing hemoglobin electrophoresis, Coulter counter (Coulter Electronics, Hialeah, FL) cell sizing, quantitative HbA_2 determination, and family studies. All patients had experienced typical vasoocclusive crises. All were in good health at the time of blood donation, and none had been transfused in the preceding 4 months.

Red cell preparation. Blood was drawn into heparinized syringes, and the red cells were separated from leukocytes and platelets by differential centrifugation at 1,000 g for ten minutes. RBC were washed four times in phosphate-buffered saline, 0.1 g/dL albumin, 0.1 g/dL glucose, 295 mosm, pH 7.4, for all of the studies

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except endothelial adherence where Hanks' balanced salt solution (HBSS) was used. Washed RBC were adjusted to approximately 10% hematocrit and were oxygenated by exposure to room air for 30 minutes at 37°C in a swirling bulb tonometer. Some cell samples were further treated with 100% CO for an additional 30 minutes at 37°C.

Fluosol and components. Fluosol DA, 20% (Fluosol), and its detergent component, Pluronic F-68 (Pluronic), were supplied by Alpha Therapeutics, Los Angeles. The Fluosol preparation was stored at −20°C and was thawed just prior to reconstitution and use. Fluosol was reconstituted according to the manufacturer's instructions and had a final composition of perfluorodecalin, 14.0 wt/vol%; perfluorotripropylamine, 6 wt/vol%; Pluronic, 2.72 wt/vol%; hydroxyethyl starch, 3.0 wt/vol%; yolk phospholipid, 0.4 wt/vol%; glycerol, 0.8 wt/vol%; NaCl, 0.6 wt/vol%; $CaCl_2$, 0.03 wt/vol%; $MgCl_2$, 0.02 wt/vol%; glucose, 0.18 wt/vol%; KCl, 0.034 wt/vol%; and $NaHCO_3$, 0.21 wt/vol%. Pluronic F-68 (Wyandotte Chemical Corp, Wyandotte, MI) was greater than 99.9% pure and was dissolved in HBSS to a final concentration equal to that in the Fluosol preparation, 2.72 wt/vol%. The washed and liganded RBC suspensions were incubated with the Fluosol or Pluronic preparations for 30 minutes at room temperature at a 5-vol/vol% (1 vol Fluosol or Pluronic in 19 vol red cell suspension) or 15 vol/vol% concentration.

Cell size, shape, and density. The hemoglobin concentration was determined spectrophotometrically by the cyanomethemoglobin method (Sigma Chemical Co, St Louis). Hematocrit (HCT) was measured by high-speed centrifugation of capillary tubes using an Adams Readacrit Microhematocrit centrifuge (Becton Dickinson, Parsipanny, NJ), and RBC counts were performed by a Coulter counter. The mean cell volume (MCV) was determined by dividing the HCT by the RBC count, and the mean cell hemoglobin concentration (MCHC) was calculated from the hemoglobin concentration and HCT. RBC density distribution was evaluated by visualization of eight-layer discontinuous dextran 40 density gradients centrifuged at 90,000 g for 90 minutes at 4°C.[23,24] The concentration of the Dextran 40 in the gradient layers varied from 24 to 38 g/dL (density, 1.078 to 1.20 g/mL). RBC morphology was evaluated by light microscopy of cell samples fixed by the sequential addition of 0.1% glutaraldehyde followed by 3.0% glutaraldehyde in White's saline, pH 7.3,[25] and by visualization of native cells in the video microscope of the micropipette apparatus. ISC were defined as cells with approximate axial-to-width ratios of greater than 2:1 after oxygenation.

Red cell filtration. A dilute suspension of washed erythrocytes was pushed at a constant flow rate through Nuclepore "Hemafil" membranes (Nuclepore Corp, Pleasanton, CA) containing cylindrical pores of approximately 5-μm diameter housed in an apparatus previously described.[26] The multiply washed RBC suspensions had WBC counts less than 100/μL. Untreated cell suspensions and RBC in 5 and 15 vol/vol% Fluosol or Pluronic were adjusted to 10% HCT and pushed through the filters at a flow rate of 1.7 ml/min. The same lot number of filters (54F0C136) was used for all of the studies.

The pressure generated across the filter during flow was continuously monitored and was used to define an index of cellular deformability as proposed by Schmalzer et al.[27] The initial pressure increment (Pi) generated by the cell suspension transversing the filter divided by the pressure increment due to fluid without cells (Po) has been experimentally and theoretically related to cellular deformability.[24] Increases in pressure after Pi are generally attributed to progressive pore obstruction due to nondeformable or permanently adhesive cells.[28,29]

Micropipette static rigidity. The static rigidity of the red cell was studied by aspiration of a small portion of the membrane into 1-μm-diameter micropipettes at pressures between 200 and 700 dyne/cm^2.[30-33] The apparent extensional shear elastic modulus (μ) of the membrane was obtained by linear regression analysis of the experimental pressure-extension length pairs utilizing the mathematical relationship described by Chien et al.[30] The derived μ reflects membrane properties in red cells of normal density and shape, but at the extremely high hemoglobin concentrations found in dense sickle cells, the internal milieu dominates the elasticity of even small micropipette deformations.[6] In this report, μ is used as an index of the static rigidity of the cell. The RBC were studied while inside a 1-mm-high glass cave, similar to that reported by Linderkamp and Meiselman.[32] The same pipette was used for paired untreated and treated RBC because of the large error introduced into the analysis by erroneous measurement of pipette radius. The effect of Pluronic was evaluated after aspiration of the untreated cells and was analyzed by use of an elastic index defined as the ratio of the μ of treated RBC to the μ of paired, untreated cells measured with the same pipette.

Endothelial adherence. RBC were examined for adherence to confluent primary cultures of human umbilical vein endothelium as previously described.[12,16] Briefly, ^{51}Cr-labeled RBC were washed and suspended to a 25% HCT in the medium in which adherence studies were conducted. They were then gently layered on endothelium and allowed to incubate for 30 minutes at 37°C. The endothelium was then washed seven times using the original suspending medium and twice with distilled water. The radioactivity in each wash was measured, and the percentage of cells remaining after each wash was determined.

Endothelial adherence was evaluated with RBC suspended in either HBSS containing 0.5% albumin or in ABO- and Rh-compatible human plasma. HBSS/albumin or plasma was altered by dilution with 5 vol/vol% Fluosol, the Pluronic alone, or HBSS (for controls). For some studies, either RBC or endothelium were pretreated with plasma containing 5% Pluronic for 20 minutes followed by five washes with plasma without Pluronic; adherence studies were then done with RBC suspended in the control plasma.

RESULTS

Filtration. Washed and oxygenated sickle red cells were considerably less filterable than normal red cells. Sickle cells generated Pi/Po ratios of 24.1 ± 8.6 and wall shear stresses of 980 to 2,940 dynes/cm^2 in filtration through the 5-μm-diameter pores. Normal cells had Pi/Po ratios of only 1.9 ± 0.3 and shear stresses approximating 200 dyne/cm^2 at the same flow rate and HCT ($P < .001$). The large variations in the sickle cell filtration pressures were due to differences between patients; the variation within a set of runs on an individual patient was less than 10%. Fluosol reduced the Pi/Po ratio of conventionally oxygenated sickle cells by one third as shown in Fig 1 but did not have a significant effect on the pressure of normal cells under the same filtration conditions (results not shown). Exposure of oxygenated sickle cells to CO did not alter filtration pressure, although CO did reduce the ISC count to 50% of that observed after conventional oxygenation. Fluosol reduced the filtration pressure of CO-exposed sickle cells as much as oxygenated cells, and Pluronic was as effective as Fluosol in improving the filtration of CO-exposed sickle cells. A 15-vol/vol% concentration of Fluosol did not improve filtration more than the 5-vol/vol% concentration (data not shown). Although the number of sickle cell patients is too small for valid statistical comparison, the patients with the highest ISC counts seemed

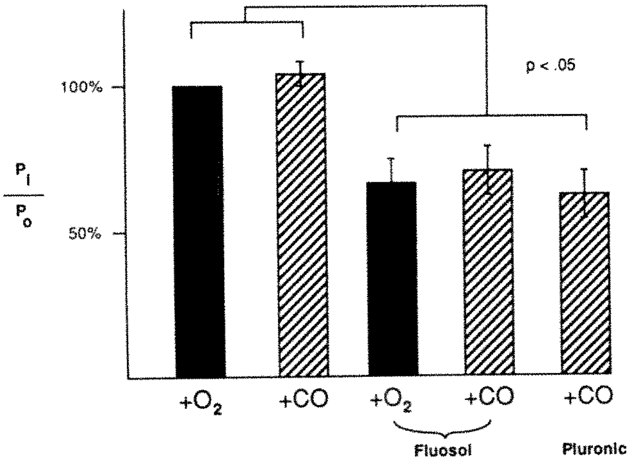

Fig 1. Both Fluosol and Pluronic improved the filtration of oxygenated (O_2) and CO-treated sickle cells. Pi/Po is a dimensionless pressure parameter defined in the text. The Pi/Po of oxygenated sickle cells has been defined as 100%; all other treatments have been normalized to the percentage of the oxygenated cell sample because of the large variability in Pi/Po between sickle cell patients. Concentrations of 5 vol/vol% of Pluronic and Fluosol were used in the studies. Each bar graph represents the mean ± 1 SD of duplicate to triplicate filtration runs from seven patients with sickle cell anemia.

to have the greatest filtration pressures and most marked pressure decrements after Fluosol or Pluronic treatment. The four patients with the highest ISC counts (mean, 20% ± 6%) showed a 41% ± 8% decrease in the Pi/Po ratio with Fluosol treatment, yet three other patients with mean ISC counts of 7% ± 3% showed only a 15% reduction in pressure.

The filtration of red cells is a complex biophysical process dependent on cellular geometry, availability of surface area for deformation, cell-to-pore size ratio, cytoplasmic and membrane viscoelasticity, and adhesive interactions with the filter. We studied the possible mechanisms contributing to the improved filtration by evaluating the influence of Fluosol and Pluronic on more independent measures of the individual biophysical factors.

Cell geometry and density. Neither Fluosol nor Pluronic had an obvious effect on cell shape or cell volume. The morphology of Fluosol- and Pluronic-treated sickle and normal red cell suspensions was indistinguishable from untreated samples in the native state under the videomicroscope and by light microscopy of glutaraldehyde-fixed preparations. The MCVs of the Fluosol (87 ± 3 fL), Pluronic (86 ± 4 fL) and untreated sickle red cell suspensions (85 ± 4 fL) were very similar. Cell density is the most important determinant of the cytoplasmic viscoelasticity of liganded sickle red cells. The MCHC of sickle cells treated with Fluosol (34.8 ± 1.1 g/dL) or Pluronic (34.1 ± .9 g/dL) did not differ from untreated cells (34.6 ± 0.8 g/dL). The distribution of the cell density of normal and sickle cells was also not altered by Fluosol or Pluronic, as illustrated by the density gradients shown in Fig 2. Hemolysis was less than 1% after exposure to either preparation over the one- to four-hour time periods encountered in the course of the studies.

Micropipette aspiration. The static rigidity of sickle and normal erythrocytes was evaluated by aspiration of small portions of the cell into 1-μm-diameter micropipettes at low pressure, which yielded an extensional shear elastic modulus (μ) that increases with greater cell rigidity. The μ of the control untreated normal RBC was $6.8 \pm 0.9 \times 10^{-3}$ dyne/cm^2; untreated discocytic sickle RBC and ISC were 8.5 ± 2.0 and $14.9 \pm 5.1 \times 10^{-3}$ dyne/cm^2, respectively. The influence of a 5-vol/vol% concentration of Pluronic on the μ of normal and sickle RBC is shown in Fig 3. Pluronic had no significant effect on the μ of normal or discocytic sickle RBC but caused a 25% reduction in μ (improvement in extensibility) in ISC.

Endothelial adherence. The influence of Fluosol and Pluronic on RBC adherence was studied by determining the number of cells attaching to endothelial monolayers under gravity sedimentation. Figure 4 depicts two representative endothelial adherence studies. The addition of a 5-vol/vol% concentration of Fluosol to HBSS/albumin completely abol-

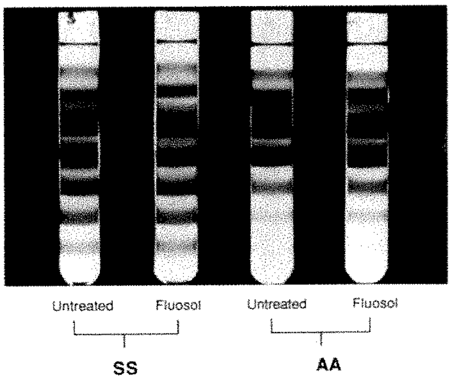

Fig 2. A concentration of 5 vol/vol% of Fluosol did not alter the distribution of cell density of normal or sickle red cells. The cell density distribution profiles of normal red cells (left) and sickle red cells (right) are shown on eight-layer discontinuous Dextran 40 gradients varying in density from 1.078 to 1.20 g/mL. Sickle cell suspensions show greater heterogeneity in cell density than normal, with increased numbers of both light and dense cells.

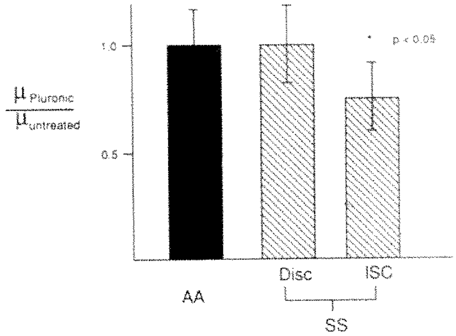

Fig 3. Pluronic reduced the static rigidity of ISC on micropipette aspiration but had no effect on normal cells or discocytic sickle cells. The effect of a 5-vol/vol% concentration of Pluronic is presented as the ratio of treated to untreated extensional shear elastic modulus (μ). An equal number of Pluronic-treated cells were studied with the same pipette used initially to aspirate untreated cells. The bar graphs represent the mean ± 1 SD of 60 normal cell pairs, 79 discocytic sickle cell pairs, and 47 ISC pairs.

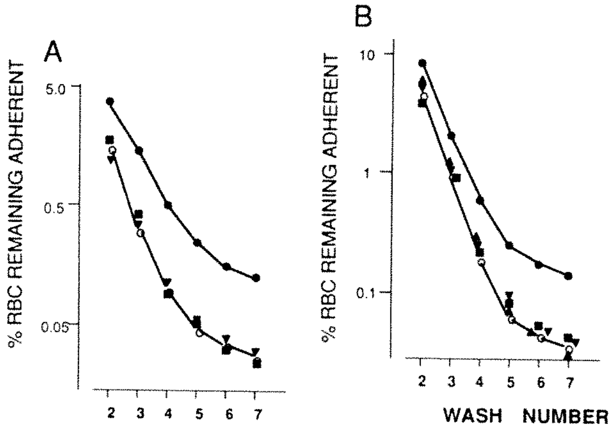

Fig 4. Fluosol and its Pluronic component abolish the abnormal adherence of sickle RBC to endothelium. Two representative studies are shown using RBC from two different sickle patients. (A) Normal RBC (open symbols) and sickle RBC (closed symbols) examined in HBSS/albumin containing 5 vol/vol% Fluosol (▼) or Pluronic (■) or HBSS as control (○, ●). (B) Normal RBC (open symbols) and sickle RBC (closed symbols) examined in plasma containing Pluronic (■) or HBSS as control (○, ●). Pretreatment of either sickle RBC (▼) or endothelium (▲) with Pluronic has the same effect as Pluronic being present during the adherence assay.

ished the abnormal adherence of sickle RBC to endothelial cells, and this effect was seen when using the Pluronic component of Fluosol alone (Fig 4A). Identical results were obtained upon examination of RBC from four additional sickle cell anemia patients. As shown in Fig 4B, the adherence-abolishing activity of the Pluronic was also evident when adherence was studied in plasma. In addition, pretreatment of either RBC or endothelium with Pluronic was just as effective in eliminating abnormal sickle adherence as was having Pluronic present during the actual adherence assay (Fig 4B).

DISCUSSION

The rheology of liganded sickle red cells was improved by the Pluronic F-68 component of Fluosol via a non-oxygen-dependent mechanism. Pluronic F-68 was as effective as Fluosol in reducing the filtration pressure of washed oxygenated and CO-treated sickle cells. Reindorf et al also found that a perfluorotributylamine preparation emulsified with Pluronic F-68 reduced the relative resistance of washed sickle cells.[22] A 20-vol/vol% admixture with deoxygenated sickle cells decreased filtration resistance even below that manifested by sickle cells equilibrated with ambient oxygen. Although the benefit was attributed to hyperoxygenation and increased hemoglobin liganding, our studies show that some of the improvement was mediated solely by the Pluronic component of the perfluorochemical.

Pluronic F-68 was also as effective as Fluosol DA in abolishing the adherence of sickle cells to endothelial monolayers. Pluronic increased the ease of aspiration of ISC into micropipettes but had no effect on the aspiration of discocytic sickle cells. Sickle cell morphology, cell volume, internal hemoglobin concentration, and distribution of cell density were not altered by exposure to Pluronic. The dramatic influence of Pluronic on endothelial cell adherence and the lack of effect on sickle cell geometry and density suggest that the improved rheology of sickle cells may be mediated by the reduction in cell adhesion. Although rigid ISC are somewhat less adhesive to endothelial cells than less-distorted sickle poikilocytes,[15] the strongly forced contact of ISC with the filter pore or glass micropipette may allow multiple point attachments to form by overcoming physical inflexibility. The shear stresses generated in sickle cell filtrations were over 1,000 dyne/cm^2, and even normal red cells adhere to the pore orifice at shear stresses above 200 dyne/cm^2.[34] Furthermore, unlike normal or discocytic sickle cells, ISC demonstrate hysteresis during micropipette aspiration that is compatible with cell adhesion to the pipette.[6]

Although a reduction in cell adhesion could explain all of the experimental observations, the sickle cell filtration and micropipette studies may also indicate an improvement in ISC deformability. The filtration behavior of sickle cell suspensions is dominated by the most rigid cellular elements, the ISC. The micropipette aspiration studies are compatible with an improvement in ISC deformability, and softening of ISC would be expected to reduce filtration pressure.

Fluosol DA and Pluronic had no effect on the filtration, adhesion, or micropipette aspiration of normal adult red cells. In contradistinction to sickle cells, normal red cells are minimally adherent to endothelium[27] or glass surfaces.[30] Holloway et al also did not find any effect of Pluronic on the filtration of normal red cells through 3-μm-diameter pores.[35]

Exposure of oxygenated sickle cells to CO in our studies reduced the number of ISC, which is in agreement with the report by Rogers et al,[11] but had no effect on the filtration pressure of sickle cell suspensions. We previously commented on the heterogeneity of filterability of oxygenated ISC and the difficulty in distinguishing ISC of variant filterability.[24] The current studies indicate that ISC undergoing shape reversal upon full hemoglobin liganding are either considerably more filterable than permanently deformed ISC or the cell's filterability is not altered by shape reversal.

Pluronic F-68 is a polyol nonionic surfactant used as an emulsifying, dispersing, wetting, and defoaming agent.[36] It is a long block copolymer of approximate molecular weight 8,500 composed of a central hydrophobic polyoxypropylene region flanked by repetitive polyoxyethylene hydrophiles on either side. The multiplicity of hydrophilic and hydrophobic groups and the relatively large size of the polymer suggest significant membrane interaction but poor cell penetration.

Pluronic reduced sickle cell adherence to endothelial monolayers in the presence of plasma as well as in saline. The dimunition of sickle cell adhesion was accomplished with equal ease by pretreatment of the sickle erythrocytes or the endothelial monolayer with Pluronic. The reduction of adhesion remained through five washes with plasma not containing Pluronic. The effectiveness of both red cell and endothelial cell treatment shows that only one cell surface need be altered to block adhesion. The irreversibility of the Pluronic exposure is consistent with the multiplicity of hydrophobic and hydrophilic groups in the polymer available for interaction with the cell membrane. Unlike the effect on sickle red cell adherence, irreversible inhibition of neutrophil adhesion required prolonged exposure to Pluronic for 18 hours.[37]

The reduction of sickle cell adherence at a relatively low concentration of Pluronic offers some hope for future therapeutic application in sickle cell crisis. The adherence of oxygenated sickle cells to endothelium is correlated to vasocclusive severity in sickle cell disease.[13,16-18] The concentrations of Pluronic required to precipitate plasma proteins, cause cytotoxicity of macrophages, or maximally impair neutrophil function are over four times greater than the concentration needed for abolition of sickle cell adherence.[37-40] Unfortunately, Pluronic is a potent activator of complement even at low concentrations, and adverse pulmonary reactions have been observed in humans after the clinical administration of perfluorochemicals emulsified with Pluronic.[38] It would seem prudent to evaluate other members of the Pluronic polyol family varying in molecular weight and proportion of hydrophobe/hydrophile to determine whether complement activation can be minimized with retention of surface-lubricating potency.

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A Test for Fanconi's Anemia

By James German, Steven Schonberg, Sandra Caskie, Dorothy Warburton, Catherine Falk, and James H. Ray

A simple and reliable cytogenetic test for Fanconi's anemia (FA) that is based on the hypersensitivity of FA cells to mitomycin C (MC) is described. Equal volumes of whole blood from a patient in whom the diagnosis of FA is suspected and from a normal person of the opposite sex are co-cultured in phytohemagglutinin-containing medium in the presence and absence of MC. After five days' co-cultivation, 100 quinacrine-stained metaphases from both the MC-containing and the MC-free cultures are examined for the presence of a Y chromosome using fluorescence microscopy. In all bona fide FA patients in whom testing was successful, hypersensitivity to MC was readily demonstrated by the striking deficiency of FA metaphases (0.9% to 14.9%) in the MC-containing co-cultures. In contrast, none of the three patients with Diamond-Blackfan anemia and none of the five with undiagnosed conditions reminiscent of FA exhibited hypersensitivity to MC; cells from them, from parents of FA patients, and from several normal laboratory personnel constituted approximately half of the metaphases (40.4% to 71.2%) of MC-containing co-cultures, as would be expected in the absence of hypersensitivity to MC.

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DIAGNOSIS of each of the several human genetic disorders that feature defective DNA repair, chromosome instability,[1] or both can be difficult without the aid of special laboratory tests because of the noteworthy degree of variability that exists in the clinical phenotypes. For example, in Fanconi's anemia (FA),[2] abnormalities of the thumb and radius may or may not be present and the age of onset of bone marrow failure is variable, making it difficult sometimes to distinguish FA from other hypoplastic disorders of the bone marrow. In fact, many FA patients totally lack congenital anomalies.

Diagnosing FA with certainty also poses a problem for the laboratory investigator. Several years ago a simple cytogenetic test for FA was devised for use in our research laboratory. The procedure, reported briefly in 1978,[3] is presented here in detail along with results obtained from the analysis of blood samples from 20 patients with developmental defects or hematologic abnormalities of the type that suggested FA as a diagnostic possibility, including 12 bona fide FA patients. Lymphocytes from three parents of FA patients and five normal laboratory personnel also were examined. Repeated use of the test by us and others[4] has confirmed its validity.

The test is based on the known hypersensitivity of FA cells to the bifunctional alkylating agent mitomycin C (MC).[5-7] Blood cells from a suspected FA patient are co-cultured with cells from a normal person of the opposite sex in medium containing MC. Simultaneously, a control co-culture lacking MC is set up. The proportion of male and female cells in the cultures at the termination of co-cultivation is estimated by scoring for the presence or absence of a Y chromosome in 100 quinacrine-stained metaphases. Hypersensitivity to MC, if present, is signified by the drastic reduction in the expected proportion of the patient's mitotic cells in the MC-treated co-culture. The results of the study demonstrate that cells from FA patients can be distinguished readily from those of patients with other related disorders.

MATERIALS AND METHODS

Patients. Lymphocytes from 12 FA patients, 5 male and 7 female, who had been diagnosed with certainty on the basis of clinical features and cytogenetic findings (identified as FA-1 through FA-12 in Table 1) were studied. Untreated cells from each of the patients exhibited chromosome instability of the type now known to be characteristic of FA[9] (Table 1). Also, as is characteristic of FA, chromosomal breakage and rearrangement were greatly accentuated by the addition of diepoxybutane (DEB) to the culture medium in the eight patients so tested.[10] (Several of the samples of blood examined using the MC co-cultivation test were tested simultaneously for DEB hypersensitivity, either in the laboratory of coauthor S.S. or in those of Drs R.S.K. Chaganti and A.D. Auerbach.) Three parents of FA patients also were examined by using the MC co-cultivation test (identified as FAhet-1 through FAhet-3).

Three persons with Diamond-Blackfan anemia (congenital red cell aplasia)[11] were tested (identified as DB-1 through DB-3). Thumb anomalies occur in a minority of persons with this rare condition, thus the need to distinguish it from FA as early as possible in view of the dire prognosis of FA.

Five patients with undiagnosed disorders were tested (identified as Un-1 through Un-5). In each case, certain clinical features had raised FA as a possible diagnosis. Evidence of increased chromosome instability had been reported in some (but not all) lymphocyte cultures from two of them, although the instability detected was never as extensive as that observed in FA. Four of the five undiagnosable patients were tested for DEB sensitivity by A. Auerbach, and all four exhibited an unambiguous lack of hypersensitivity like that typically found in FA (personal communication, 1987).

Finally, blood cells from five normal persons (identified as N1-1 through N1-5) were co-cultivated.

MC co-cultivation test. The patient's heparinized whole blood (0.5 mL) was placed into each of two culture vessels containing 10 mL of medium (Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1% phytohemagglutinin [PHA]); one of the two vessels also contained 25 ng/mL MC (ICN Pharmaceuticals, Inc,

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Table 1. Clinical and Cytogenetic Features of 28 Persons Examined for Hypersensitivity to MC in the MC Co-cultivation Test

Person Tested	Age	Development Defects*	Chromosome Instability†
FA			
FA-1	19 yr	BEDFGKMPS	+ (16)
FA-2	6 yr	BEGHJKMPS	+ (29)
FA-3	2 yr	BG	+ (17)
FA-4	9 mo	CGJKNS	+ (35)
FA-5	7 yr	BEGMOPRS	+ (36)
FA-6	14 yr	BEGS	+ (15)
FA-7	2 yr	EGJPS	+ (10)
FA-8	2 mo	BGJR	+ (12)
FA-9	11 yr	BEGJMPS	+ (15)
FA-10	<8 wk	B	+ (10)
FA-11	21 mo	BGJKR	+ (9)
FA-12	15 yr	BEG	+ (15)
Parents of FA			
FAhet-1	>25 yr	O	− (11)
FAhet-2	>25 yr	O	− (8)
FAhet-3	>25 yr	O	− (11)
Diamond-Blackfan anemia			
DB-1	9 yr	BEQ	− (7)
DB-2	3 mo	BEI	− (6)
DB-3	31 yr	E	− (2)
Undiagnosed disorders			
Un-1	12 yr	EMP	− (4)
Un-2	19 yr	A	− (5)
Un-3	10 yr	BGDJRS	± (5; 14)
Un-4	36 yr	KLP	− (2)
Un-5	3 wk	BR	− (6)
Normal persons‡			
Nl-1	24 yr	O	−
Nl-2	>40 yr	O	−
Nl-3	35 yr	O	− (1)
Nl-4	27 yr	O	− (3)
Nl-5	24 yr	O	−

Blank spaces represent lacking or inconclusive data.

*A, familial cerebellar atrophy with ataxia (patient II-4[8]); B, thumb anomaly; C, clone, 8% to 15% of circulating T lymphocytes with a translocation, t(4p−;9q+); D, mental ability low, average to mildly deficient; E, anemia or depressed erythropoeisis; F, myocardial infarction at the age of 21; G, growth deficiency; H, heart anomaly; I, possible depression in myelopoeisis; J, anomaly of structure other than limb, kidney, or heart; K, kidney or ureteral anomaly; L, familial anemia; M, depression in myelopoeisis; N, neoplasia (brain tumor); O, no abnormalities, healthy; P, depression in platelet production; Q, possible heart and possible kidney anomalies; R, radius absent or hypoplastic; S, skin hyperpigmentation.

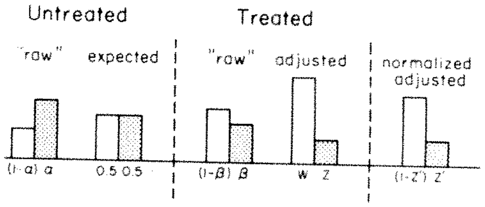
†In parentheses is shown the percentage of cells with at least one chromosomal aberration (gap, break, rearrangement) found during the analysis of 100 consecutive, well-spread, aceto-orcein-stained cells except the following: Un-3, 200 cells in first analysis; Un-5, 50 cells.

‡Tests in which lymphocytes from two normal people were co-cultivated. In all persons above this point in the Table, lymphocytes from one normal person were co-cultivated with those from a patient or a patient's parent.

Cleveland). The stock solution of MC (5 μg/mL) was prepared in Dulbecco's phosphate-buffered saline (Mg^{++} and Ca^{++} free) and stored frozen after sterilization by passage through a Millex filter (0.22 μm). An equal volume of blood from an opposite-sexed normal person also was added to each culture vessel, the object being to co-cultivate approximately equal numbers of PHA-responsive male and female lymphocytes. (Leukocyte counts were not made because of the impossibility of estimating the number of PHA-responsive T lymphocytes.) To increase the chance of success of the testing, duplicate co-cultures sometimes were set up with the same or different normal controls.

After five days' incubation (37°C, 5% CO_2), including a final hour in the presence of Colcemid (Ciba, Summitt, NJ) (6 μg/mL), the co-cultures were harvested. Metaphase preparations from the co-cultures were placed on slides and stained with quinacrine dihydrochloride.[12] The slides were coded, and 100 metaphases from each co-culture were examined with the aid of a fluorescence microscope; each metaphase was scored for the presence or absence of a Y chromosome. The proportion of male (Y-positive) to female (Y-negative) metaphases in the MC-free co-culture, never exactly a 1:1 ratio and sometimes quite far from it, was "adjusted" to give that ratio so that estimation of the true proportion of dividing male and female cells in the MC-treated co-culture would be facilitated. (See Fig 1 for the adjustment formula, its derivation, and an example of its use.) Such adjustment, though not essential for evaluating the results of an individual test, facilitates comparison of results from different tests; the data summarized in Table 3 and Fig 2 have been so adjusted.

Occasionally, metaphases from either the patient or the normal



$$Z' = \frac{\beta(1-\alpha)}{\beta(1-\alpha)+\alpha(1-\beta)}$$

Fig 1. Diagram and formula illustrating adjustment of the proportion of the patient's metaphases to the opposite-sexed control's metaphases in MC co-cultivation test. Derivation of the adjustment formula is as follows. Let M = number of FA metaphases in untreated co-culture; m = total number of metaphases of both sexes in untreated co-culture; N = number of FA metaphases in treated co-culture; and n = total number of metaphases of both sexes in treated co-culture. The proportion of FA metaphases in the untreated and treated co-cultures will be $\alpha = M/m$ and $\beta = N/n$, respectively. (Ideally, m and n will each be 100.) The untreated co-culture is a control designed to give a background estimate of the relative proportion of the two types of cells under normal conditions. Because the control seldom will be found to have the expected 50:50 ratio of normal and FA metaphases, the treated ratios are adjusted according to the deviations from 50:50. To do this the following relationships are used: $\alpha/\beta = 0.5/Z$, which gives $Z = 0.5\beta/\alpha$, and $(1-\alpha)/(1-\beta) = 0.5/W$, which gives $W = 0.5\,(1-\beta)/(1-\alpha)$. The sum of Z + W will not necessarily be 1, so a further normalizing adjustment is made:

$$Z' = \frac{Z}{Z+W} = \frac{\dfrac{0.5\beta}{\alpha}}{\dfrac{0.5\beta}{\alpha}+\dfrac{0.5(1-\beta)}{(1-\alpha)}}$$

Multiplying the numerator and denominator by $\alpha\,(1-\alpha)$ to clear the fractions and simplifying gives the adjustment formula: $Z' = \beta(1-\alpha)/[\beta(1-\alpha)+\alpha(1-\beta)]$. ($Z' \times 100$ converts the proportion to a percentage.) As an example of the use of the adjustment formula, the data for FA-11 in Table 2 were handled as follows: $\alpha = 72/100 = 0.72$; $1-\alpha = 0.28$. $\beta = 25/100 = 0.25$; $1-\beta = 0.75$. $Z' = 0.25(0.28)/[0.25(0.28)+0.72(0.75)] = 0.115$ (or 11.5%).

Table 2. Results of MC Co-cultivation Testing of the 28 Individuals Listed in Table 1

Person Tested	Metaphases Scored in Co-culture* Untreated Patient/Control	Metaphases Scored in Co-culture* MC-Treated Patient/Control	Adjusted Percentage of Patient Metaphases in MC-Treated Co-cultures†	Hyper-sensitivity to MC‡
FA				
FA-1	17/75	3/91	11.4	
	21/74	4/90	14.9	+
	42/54	3/89	4.1	+
	44/54	3/92	3.9	+
FA-2	9/91	1/69	12.8	
FA-3	90/ 8	5/95	0.6	
FA-4	77/23	3/97	0.9	+
FA-5	32/68	1/99	2.1	+
FA-6	62/38	9/91	5.7	+
FA-7	60/40	13/87	9.1	+
	52/48	15/85	14.0	+
FA-8	40/60	2/98	3.0	+
	51/49	3/97	2.9	+
FA-9	67/33	16/84	8.6	+
	80/20	19/81	5.5	+
FA-10	78/22	29/71	10.3	+
	72/28	32/68	15.5	+
FA-11	72/28	25/75	11.5	+
FA-12	20/80	1/99	3.9	+
Parents of FA				
FAhet-1	55/45	42/19	64.4	–
FAhet-2	34/66	31/69	46.6	–
FAhet-3	70/30	73/27	53.7	–
Diamond-Blackfan anemia				
DB-1	79/21	77/23	65.6	–
	77/23	78/22	51.4	–
	66/34	72/28	57.0	–
	71/29	70/30	48.8	–
DB-2	21/79	49/52	78.0	–
	35/66	29/52	51.3	–
DB-3	67/33	59/41	41.5	–
	58/42	60/40	52.1	–
Undiagnosed patients				
Un-1	43/57	60/40	66.5	–
	53/47	59/41	56.1	–
Un-2	68/32	84/16	71.2	–
Un-3	34/66	31/69	46.6	–
	27/73	33/67	57.1	–
Un-4	19/81	7/93	24.3	
	14/86	12/88	45.6	
Un-5	77/23	88/12	68.6	–
Normal persons				
Nl-1	35/61	33/64	47.3	–
Nl-2	34/63	57/35	54.6	–
Nl-3	25/75	33/67	59.6	–
Nl-4	75/25	67/33	40.4	–
Nl-5	61/35	64/33	52.7	–
	63/34	35/57	45.4	–

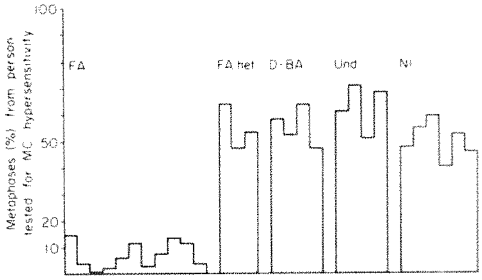


Fig 2. Graphic representation of the adjusted data summarized in Tables 2 and 3 demonstrating hypersensitivity of cells from persons with FA to MC and the lack of it in their parents, normal controls, and several patients with conditions in some way resembling FA. Each column represents the proportion of a tested person's metaphases in an MC-treated co-culture. FA, bona fide Fanconi's anemia patients; FAhet, parents of patients with FA; D-BA, patients with Diamond-Blackfan anemia; Und, patients with undiagnosed conditions in some respects resembling FA; Nl, normal laboratory personnel.

person were greatly underrepresented in the MC-free co-culture even though equal volumes of the two blood samples had been added to the culture flask. When cells from either of the partners comprised fewer than 20% of the metaphases in the MC-free co-culture, that particular test, considered to lack a suitable control, was arbitrarily invalidated.

RESULTS

When lymphocytes from normal persons were co-cultivated with those from patients in whom the diagnosis of FA had been established beyond a reasonable doubt (patients FA-1 through FA-12 in Table 1), the presence of MC in the culture medium led to a drastic underrepresentation of mitotic FA cells. Unequivocal (ie, diagnostic) results were obtained in tests of ten of the 12 FA patients (Tables 2 and 3, Fig 2): 0.9% to 14.9% of the dividing lymphocytes in the MC-treated co-cultures were derived from the FA partner. Proliferation of cells from the other two FA patients, FA-2 and FA-3, was also depressed severely in the presence of MC (Table 2), but the tests were considered invalid because of the relatively poor proliferation in the MC-free co-culture of cells from the control person in one instance and the FA patient in the other (as explained in Materials and Methods and in a footnote to Table 2). Data obtained from the study of these bona fide FA patients suggest that the results of an MC co-cultivation test should be considered positive when the patient's cells comprise fewer than 15% of the metaphases in the MC-treated co-culture.

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*In the test as performed in recent years a total of 100 metaphases are scored, the traversing of the slide being continued until that many cells are found that can be classified unambiguously with respect to the presence of a Y chromosome. Earlier, only 100 total metaphases were examined, ambiguous ones then being rejected (as, for example, when chromosomes overlap and obscure the fluorescence of individual chromosomes), leaving the number of Y-positive and Y-negative cells something less than 100 (as will be seen in certain entries in the Table). When more than one set of data appear in the table for a given patient, the data in each were derived from a separate co-culture. A brace indicates that the co-cultures were duplicates, the patient co-cultivated with the same control.

†The adjusted figures in this column represent the percentages of metaphases from the subject being tested that would have been present in the MC-treated culture had the desired 50:50 ratio of 46,XY:46,XX cells been obtained in the untreated co-culture adjusted as in Fig 1. The actual numbers of Y-positive (male) and Y-negative (female) cells scored appear in columns 2 and 3.

‡Blanks appear in this column in the case of invalid tests (see Materials and Methods). A study arbitrarily is considered valid when cells of both of the partners in the co-culture lacking MC are represented by >20% of metaphases. In two of the FA patients (FA-2 and FA-3), although MC hypersensitivity may well have been indicated by the test, there being, respectively, only 12.8% and 0.6% of their metaphases represented in the MC-containing co-cultures, their testing is viewed as invalid because in the MC-free co-cultures <20% of the metaphases were derived from the patient (only 9% for FA-2) or the control (only 8% for FA-3).

Lymphocytes from parents of patients with FA, presumably heterozygous carriers of an FA gene, exhibited no evidence of hypersensitivity to MC and comprised 46.6% to 64.4% of the metaphases in MC-treated co-cultures (Tables 2 and 3, Fig 2).

Lymphocytes from patients DB-1, DB-2, and DB-3 exhibited no hypersensitivity to MC in the test: they comprised 46.8% to 64.6% of the metaphases in MC-treated co-cultures (Tables 2 and 3, Fig 2). These children are anemic, and two of the three have abnormal thumbs. They manifest no chromosome instability and clinically are considered to have Diamond-Blackfan anemia rather than FA (Table 1).

Each of the patients identified as Un-1 through Un-5 was and remains a diagnostic problem (Table 1). On clinical grounds FA seems an unacceptable diagnosis in any one of them, even though it was considered as a possibility in their differential diagnoses. Lymphocytes from those undiagnosed patients in whom valid test results were obtained exhibited no hypersensitivity to MC as determined by the co-cultivation test: they comprised 51.8% to 71.2% of the metaphases in MC-treated co-cultures (Tables 2 and 3, Fig 2). Patient Un-5 is an infant born lacking both thumbs and one radius, but otherwise she is healthy; although her anomalies are unexplained, she, in contrast to FA-10 who has similar anomalies, is considered not to have FA on the basis of the demonstration of normosensitivity to MC (and in testing elsewhere to DEB).

Lymphocytes from five normal persons were co-cultivated in various combinations, and in no instance did normal cells exhibit hypersensitivity to MC (Tables 2 and 3, Fig 2). (In addition to the testing described in this report, valid tests have been made in three patients with Bloom's syndrome and one with dyskeratosis congenita, none of whom exhibited hypersensitivity to MC.)

DISCUSSION

The results presented here demonstrate that MC co-cultivation is a useful tool for the differential diagnosis of FA. Several laboratories besides ours have occasion to test for FA on a regular basis. Each laboratory has its own test system for determining whether the diagnosis of FA is to be made. The laboratories of M.S. Sasaki in Kyoto (personal communication, 1986) and of J. Cervenka in Minneapolis[13] test for excessive chromosome breakage in the patient's cells after exposure to MC. Those of T.M. Schroeder in Heidelberg,[14] R. Berger in Paris,[15] and A. Auerbach in New York[10] test for excessive chromosome breakage after treatment of the cells with other chromosome-damaging chemicals such as isonicotinic acid hydrazide (INH), nitrogen mustard, and DEB. The laboratory of S.A. Latt in Boston uses a test based on MC-induced changes in DNA histograms derived by fluorescence flow cytometry, the MC treatment causing an excessive accumulation of FA cells in the late S or G_2 phase of the cell cycle.[16,17] That of H. Hoehm in Würzburg also uses flow cytometry, but to demonstrate excessive arrest in G_2 of PHA-stimulated lymphocytes even when not treated with MC.[18] Results from these laboratories as well as our own indicate that, in experienced hands, each of the tests is useful and accurate in the identification of persons with FA. However, the clinical diagnostic laboratory that will be called upon only occasionally to assist in diagnosing FA will find that the MC co-cultivation test described here has the advantages of requiring neither personal knowledge on the part of the microscopist of the morphological effects on metaphase chromosomes of MC, INH, nitrogen mustard, or DEB nor access to a flow cytometer. With this test, any cytogenetics laboratory experienced in classifying metaphases as to sex chromosome constitution—here we use fluorescence microscopy to score for the presence or absence of the distinctively brightly fluorescent Y chromosome—can make a straightforward examination of 100 metaphases in the MC-treated co-culture directly through the microscope and in a short time arrive at a conclusion as to the presence or absence in the patient of hypersensitivity to MC, provided only that adequate numbers of lymphocytes from both the patient and the control divide in the MC-free co-culture.

It is possible that genetic disorders other than FA will be recognized in the future that feature hypersensitivity to MC. Conceivably, some people in the general population will be detected who are hypersensitive to MC. Therefore, the demonstration of hypersensitivity to MC in the absence of clinical features of FA in patients or in one of their siblings cannot be said to diagnose FA. Nevertheless, the simple and reliable test for hypersensitivity to MC described here is very

Table 3. Summary of Results of MC Co-cultivation Testing of the 28 Individuals Studied

Clinical Condition	No. of Persons Tested	Persons With Valid Tests	Persons With Invalid Tests	Percentage of Test Cells in MC-Treated Co-culture* Range (%)	Percentage of Test Cells in MC-Treated Co-culture* Mean (%)	No. Persons Hypersensitive/ No. Valid Tests
FA	12	10	2	0.9-14.9	7.0	10/10
Parents of FA	3	3	0	46.6-64.4	54.9	0/3
Diamond-Blackfan anemia	3	3	0	46.8-64.6	55.6	0/3
Undiagnosed patients	5	4	1	51.8-71.2	63.2	0/4
Normal persons	5	5	0	40.4-59.6	50.0	0/5

*When valid duplicate test results were available (see footnote, Table 2), the averages were used here and in Fig 2.

useful in the evaluation of persons suspected on clinical grounds of having this rare genetic disorder. Furthermore, the use of this or some other procedure for examining sensitivity to MC in patients with unexplained anemias, granulocytopenias, thrombocytopenias, and anomalies of the thumb and radius should hasten a definition of the entire clinical spectrum of FA. If the clinical features presented by the patient are either suggestive of or compatible with FA and the results of the MC co-cultivation test are positive, then the diagnosis of FA can be made with confidence.

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A Randomized Prospective Comparison of Chemotherapy to Total Body Irradiation as Initial Treatment for the Indolent Lymphoproliferative Diseases

By Peter Jacobs and Helen S. King

One hundred eight consecutive patients with indolent lymphoproliferative diseases were stratified into chronic lymphocytic leukemia (CLL), stage III and IV well-differentiated lymphocytic lymphoma (WDLL), and stage III and IV follicular lymphoma (FL). Within each stratum, patients were prospectively and randomly assigned to receive chemotherapy with chlorambucil and prednisone (CP) or fractionated total body irradiation (TBI). Morbidity from both regimens was negligible. Complete response (CR) was defined as the resolution of organ enlargement and the return of blood count to normal. The CR rate for the entire CP group (n = 54) was 59% and that for the TBI group (n = 54), 52%; median survivals were 53 and 57 months respectively. In the 41 patients with CLL the CR rate for CP (n = 17) was 47% and that for TBI (n = 24), 50%; the median survival for CP was 48 months, and for TBI it was 51 months. In the 21 patients with WDLL the CR rate for CP (n = 15) was 53% and that for TBI (n = 6), 67%; the median survival for CP was 42 months and has not yet been reached for TBI. For the 46 patients with FL the CR rate for CP (n = 22) was 72% and that for TBI (n = 24), 50%; the median survival was 55 months, and for TBI it was 56 months. None of the differences in CR or survival are statistically significant ($P > .05$). In these indolent lymphoproliferative diseases, CP and TBI are equally effective forms of initial treatment irrespective of the end point being defined as CR or survival.

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THE INDOLENT B lymphoproliferative diseases are conventionally classified on the basis of clinical and hematologic features into chronic lymphocytic leukemia and the low-grade non-Hodgkin's lymphomas. However, immunologic evidence favors their inclusion within a single spectrum in which differing presentations are explicable on the point at which B cell maturation is disturbed.[1] Accordingly, all individuals with such neoplasms can reasonably be included in studies to evaluate response to therapy, although stratification into their separate morphological entities is appropriate.

Where treatment is considered necessary, experience accumulated in the last 20 years with chlorambucil and prednisone(CP)[2,3] provides a standard against which alternative options can realistically be compared. Initial response rates are generally over 50%, and there appears to be merit in using pulsed as opposed to continuous therapy in attempts to protect hematopoietic reserve, but the emergence of drug resistance and the development of side effects due to the alkylating agents and prednisone are acknowledged hazards. An alternative treatment is total body irradiation (TBI),[4] which prolongs survival, returns performance status to normal, and may be associated with recovery of immunoglobulin levels.

Neither chemotherapy nor radiotherapy is entirely benign, and both may be associated with morbidity and mortality. However, these two treatment options have been compared in chronic lymphocytic leukemia (CLL)[5] and favorable-histology non-Hodgkin's lymphoma.[6] Although differences occurred between the two forms of treatment, the median duration of survival was comparable.

To further characterize response rate and survival, a prospective randomized trial was undertaken to compare CP with TBI in a consecutive series of previously untreated patients with low-grade lymphoproliferative diseases. In this study the opportunity existed to examine potentially relevant prognostic factors including nutritional status, late presentation, and ethnic differences.

PATIENTS AND METHODS

Stratification. One hundred eight consecutive adults with chronic lymphoproliferative disease were stratified into CLL (n = 41), stage III or IV well-differentiated lymphocytic lymphoma (WDLL) (n = 21), and stage III or IV follicular lymphoma, including all cell types (FL) (n = 46).

Staging. All patients had a detailed clinical assessment by history and physical examination followed by chest x-ray. Established and standardized methods were used for biochemical profile and peripheral blood examination, bone marrow aspiration, and trephine biopsy.[7,8] Anemia and thrombocytopenia were evaluated by appropriate additional studies, including the Coombs' test and the platelet membrane-associated immunoglobulin test. Nutritional status was assessed by total protein and albumin levels, iron profile, serum and red cell folate and vitamin B_{12} levels. Wherever superficial lymph nodes were palpable, these were removed for morphologic, histopathologic, and cell membrane surface marker studies.

Patients with CLL were staged according to the classification of Rai et al[9] and those with lymphocytic lymphoma according to the Ann Arbor modification of the Rye recommendation.[10]

Trial design. The patients were prospectively entered in a protocol study approved by institution review boards, and participation required informed consent. After confirmation of diagnosis, individuals were randomly assigned to receive CP or TBI.

Complete response (CR) was defined as the disappearance of all clinically evident disease, with normal hemoglobin, white cell, and platelet counts. Persisting peripheral blood lymphocytosis up to 50% and 25% residual lymphocytes in the marrow were acceptable within this definition. The term complete remission, as used in some

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publications, was not considered appropriate, since immunologic evidence for eradication of the abnormal clone was not obtained.

Chlorambucil therapy was started at an oral dose of between 0.15 and 0.20 mg/kg (usually 12 mg orally each day) and given to hematologic tolerance defined as a platelet count of 100 × 10^9/L and a granulocyte count of 2 × 10^9/L. For the first month, prednisone was given orally at a dose of 0.5 mg/kg, and then treatment was discontinued.

The TBI was given with a cobalt 60 unit at a rate of 13 to 17 cGy/min on a fractionated schedule of 15 cGy twice a week to a total of 150 cGy. Further treatment was discontinued if the platelet count fell below 100 × 10^9/L or granulocytes below 2 × 10^9/L.

Patients on the chemotherapy arm who achieved CR continued daily chlorambucil treatment at the lowest dose required to prevent disease relapse. Those who failed to meet the criteria for CR or who relapsed were removed from the chemotherapy arm and were treated with TBI. Patients relapsing from radiotherapy were offered a second course of TBI, after which they crossed over to CP.

All patients were eligible to receive supportive treatment, which included intensive management with antibiotics for infection, platelet or red cell transfusion, and in selected individuals, splenectomy for control of pancytopenia. Immune thrombocytopenia or hemolysis was treated with additional courses of prednisone, and in refractory cases, by splenectomy.

Statistics. Response rates were compared for statistical significance by using Yates' correction of the chi-square test. Survival curves were drawn by the Kaplan-Meier life table method and compared by using the log rank test.[11]

RESULTS

Patients. The age, sex, and ethnic distribution of the patients in the three groups is shown in Table 1. The ratio of white patients to those of mixed blood ancestry[12] and of black descent approximates the referral pattern to both the hospital in general and the hematology clinic more specifically.

Nutritional status and initial biochemistry. There was no significant difference in total protein, albumin, iron folate, or vitamin B_{12} levels or in the median values for uric acid, urate, creatinine, bilirubin, lactic dehydrogenase, calcium, or phosphorus between patients in either treatment arm of the three disease subgroups or when those of different ethnic origins are compared.

Table 1. Age, Sex, and Ethnic Distribution

Patient	Disease Classification CLL	WDLL	FL
Age			
Median (yr)	58	60	51
Range (yr)	42-80	44-73	30-70
Sex, male:female			
Total Group	29:12	12:9	24:22
White	20:7	7:5	15:16
Mixed blood ancestry	7:5	4:4	8:6
Black	2:0	1:0	1:0
Ethnic distribution			
White	27	12	31
Median age (yr)	61	61	59
Range (yr)	43-76	44-72	30-70
Mixed blood ancestry	12	8	14
Median age (yr)	56	56	59
Range (yr)	42-77	44-73	45-64
Black	2	1	1
Median age (yr)	68	55	43
Range (yr)	67-80		

The ethnic distribution of patients in this study approximates the referral pattern to both Groote Schuur Hospital and more specifically to the Haematology Clinic.

Table 2. Presenting Hematologic Features

Laboratory Measurements	Disease Classification CL (n = 41)	WDLL (n = 21)	FL (n = 46)
Hemoglobin (g/dL)			
Median	13.4	12.7	13.7
Range	5.1-17.2	6.9-16.6	7.1-18.1
Total white cell count (× 10^9/L)			
Median	34.8	9.3	6.4
Range	5.9-41.6	4.2-25.4	3.0-31.4
Platelet count (× 10^9/L)			
Median	200	242	262
Range	104-387	105-567	109-587

Apart from the total white cell count and the percentage of peripheral blood lymphocytes there are no significant differences in hematology at presentation between the three disease groups.

Presenting hematology. Median pretreatment values and range are presented in Table 2.

Clinical staging. The clinical stages of patients in each stratum are presented in Table 3.

Toxicity. Neither the CP nor the fractionated TBI was associated with mortality, and the morbidity from both regimens was negligible. No cases of acute leukemia have occurred.

Comparison of CP with TBI. When the patients from the three groups are pooled, the CR rate for those receiving CP (n = 54) was 59%, and that for the TBI group (n = 54) was 52%; median survivals were 53 and 57 months, respectively. The overall survival is not significantly different ($P > .05$).

CLL (n = 41). Immunologic marker studies were available on 88% of the patients. Characteristically, rosette formation with mouse erythrocytes was present. With the exception of two patients, all expressed a B lymphocyte phenotype; in two, marking was indistinct, but no patients had demonstrable T lymphocyte cell membrane surface markers. Two patients expressed IgG κ, one IgM λ, and the remainder, IgM κ.

Table 3. Clinical Staging of Patients

Disease	Stage 0	I	II	III	IV
CLL (n = 41)	4	15	14	4	4
WDLL (n = 21)	0	0	0	3	18
FL (n = 46)	0	0	0	14	32

CLL was staged according to the recommendations of Rai et al[9] and WDLL and FL, according to the Ann Arbor modification of the Rye recommendation.[10]

Table 4. Response to Therapy

	Disease Classification		
	CLL (n = 41)	WDLL (n = 21)	FL (n = 46)
CP group (n)	17	15	22
CR	8 (47%)	8 (53%)	16 (72%)
Median duration (mo)	23	33	26
CCR	4	3	6
Relapsed	4	5	10
Interval (mo)	13-29	19-66	4-38
Failed	9	7	6
TBI group (n)	24	6	24
CR	12 (50%)	4 (67%)	12 (50%)
Median duration (mo)	21	14	6
CCR	6	0	1
Relapsed	6	4	11
Interval (mo)	5-60	9-26	4-36
Failed	12	2	12

The CR rates are given in absolute numbers and percentages. These data are further analyzed to show CCR and the numbers of patients who have relapsed.

The response to therapy is summarized in Table 4. The median survival for the CP group (n = 17) was 48 months and for the TBI group (n = 24), 51 months. The survival curves for these two forms of therapy are not significantly different ($P > .05$) (Fig 1).

WDLL (n = 21). Immunologic marker studies showed only B lymphocyte phenotypes.

The response to therapy is summarized in Table 4. The median survival for the CP group (n = 15) was 42 months, and that for the TBI group (n = 6) has not yet been reached. The survival curves for these two forms of therapy are not significantly different ($P > .05$) (Fig 2).

FL (n = 46). Immunologic marker studies showed only B lymphocyte phenotypes.

The response to therapy is summarized in Table 4. The median survival for the CP group (n = 22) was 55 months, and that for the TBI group (n = 24) was 56 months. The survival curves for these two forms of therapy are not significantly different ($P > .05$) (Fig 3).

Miscellaneous. Immunologic marker studies within each of the three strata showed no phenotypic difference between the CP and TBI patients or the racial groups studied. In none of the analyses was there any correlation between the pattern of bone marrow involvement or response to either form of therapy.

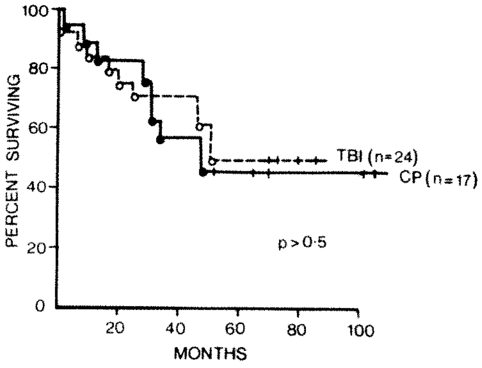

Fig 1. Survival curves for patients with CLL. In the CP group the median survival has not yet been reached, and in the TBI group it is 50 months; these differences are not statistically significant ($P > .05$).

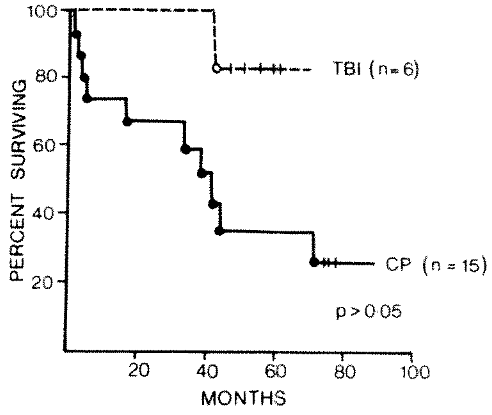

Fig 2. Survival curves for patients with WDLL. In the CP group the median duration is 44 months, and in the TBI group survival has not yet been reached; these differences are not statistically significant ($P > .05$).

Morphological concordance between lymph node biopsy and bone marrow cytology was not possible in patients with CLL and WDLL due to the paucity of material. However, in patients with FL, sufficient material was available to compare lymph node histopathologic features with marrow cytology. Analysis of 32 of 46 patients showed a concordance in 21 but a discordance in 11 where mixed large and small cells were present in the node, but only small cleaved cells were demonstrable in the marrow; this pattern was comparable between the CP and TBI groups.

DISCUSSION

In this randomized and prospective clinical trial, chemotherapy with CP gave comparable results to TBI in the response rate and survival for patients with the indolent lymphoproliferative diseases defined to include CLL, WDLL, and FL. Neither form of therapy was associated

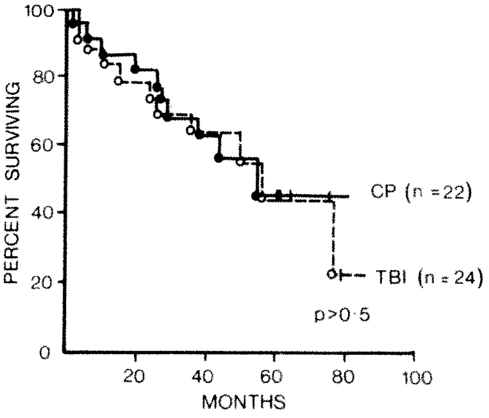

Fig 3. Survival curves for patients with FL. The median survival for both groups is 54 months, and these curves are not statistically significant ($P > .05$).

with any unacceptable degree of patient morbidity, no patient died from either CP or TBI, and to date no cases of acute leukemia have occurred.

When CP and TBI are contrasted in the context of the chronic lymphoproliferative disorders, radiotherapy emerges with two apparent attractions. First, there is generally prompt and often dramatic resolution of enlarged organs, notably the superficial lymph glands; although this was not reflected in better survival than with the use of CP, patients found this reassuring. Second, in those individuals who travel long distances to be treated or where compliance was unpredictable, TBI provided an effective form of therapy, equivalent to conventional CP but without the need for frequent follow-ups to monitor blood counts or concern about failure to take medication.

One additional point became clear from this study. Although TBI is an excellent form of initial therapy for patients with CLL and low-bulk lymphoma, the response was less satisfactory in those with large volumes of tumor, which in our experience was restricted to FL. Thus, in this latter subgroup of patients, residual tumor masses remained present in five of the 24 and required additional local radiotherapy for disease control, which resulted in troublesome pancytopenia, with four patients becoming transfusion dependent and one developing thrombocytopenia. With the exception of this specific patient population, myelotoxicity has been no more of a problem with TBI than CP and therefore differs from some of the other radiotherapy results reported.[13,14]

Further analysis of the data from this study is conveniently based on the stratification into the three clinically distinct entities.

CLL was classified on the basis of the original staging of Rai et al,[9] and although this latter approach is recognized to have some deficiencies,[15] there were also no statistically significant differences between CP and TBI when using the recommendations of the international workshop. Furthermore, although the pattern of bone marrow involvement is said to be of prognostic significance[8] there was no clear-cut relationship demonstrable with clinical stage or response to therapy.

WDLL accounts for only 5% of indolent lymphomas and, like CLL, frequently has bone marrow involvement,[16,17] but also expresses immunologic and phenotypic differences.[18] Not surprisingly, therefore, CP was therapeutically equivalent to TBI, as previously reported,[5] although long-term hematologic depression was not more commonly encountered in our patients receiving TBI. We also observed that the period of treatment required to achieve a response was shorter with TBI, and although late relapses occurred with both forms of therapy, the median survival was equivalent and protracted. The uneven randomization requires comment and is accounted for by four patients. In each of these it was necessary to use chemotherapy after they were randomized, since three found the positioning for the radiotherapy uncomfortable and in the fourth the isolation of the treatment room proved insurmountable.

The FLs, with the exception of the nodular histiocytic (large cell) variant, are generally reported to have a favorable prognosis despite advanced disease at presentation.[19] Although we were aware of the heterogenity of these tumors,[20] examination of our patient material using both the scheme of Rappaport et al[21] and the National Cancer Institute's working formulation[22] showed no difference for the patient groups receiving CP or TBI; these findings are consistent with previously reported results.[6] It is, however, noteworthy that in a small subgroup of our patients with FL having high-bulk disease, additional radiotherapy was needed to control residual tumor masses and resulted in protracted pancytopenia. With the exception of this special circumstance, the myelosuppressive effects of conventional CP and TBI were comparable.

It is concluded that patients with indolent lymphoproliferative diseases who share a common B cell phenotype will have comparable response rates and survival when treated with conventional CP and TBI. Furthermore, this equivalent outcome remains evident after stratification into CLL, WDLL, and FL. It is noteworthy that TBI, although conferring no survival advantage, resulted in a more-rapid resolution of organ enlargement and generally less need for close patient follow-up. Myelotoxicity was no greater with TBI, although patients with high-bulk FL often required additional radiotherapy for disease control, and these circumstances compromised hematopoietic tolerance for subsequent chemotherapy due to the development of pancytopenia. This latter subgroup of patients might reasonably be considered for alternative forms of initial therapy, perhaps pulses of high-dose alkylating agents or different schedules of systemic radiotherapy.

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Differences in Cell Cycle Characteristics Among Patients With Acute Nonlymphocytic Leukemia

By Azra Raza, Yogesh Maheshwari, and Harvey D. Preisler

The proliferative characteristics of myeloid leukemias were defined in vivo after intravenous infusions of bromodeoxyuridine (BrdU) in 40 patients. The percentage of S-phase cells obtained from the biopsies (mean, 20%) were significantly higher ($P = .00003$) than those determined from the bone marrow (BM) aspirates (mean, 9%). The post-BrdU infusion BM aspirates from 40 patients were incubated with tritiated thymidine in vitro. These double-labeled slides were utilized to determine the duration of S-phase (T_s) in myeloblasts and their total cell cycle time (T_c). The T_s varied from four to 49 hours (mean, 19 hours; median, 17 hours). Similarly, there were wide variations in T_c of individual patients ranging from 16 to 292 hours (mean, 93 hours; median, 76 hours). There was no relationship between T_c and the percentage of S-phase cells, but there was a good correlation between T_c and T_s ($r = .8$). Patients with relapsed acute nonlymphocytic leukemia (ANLL) appeared to have a longer T_s and T_c than those studied at initial diagnosis. A subgroup of patients at either extreme of T_c were identified who demonstrated clinically documented resistance in response to multiple courses of chemotherapy. We conclude that T_s and T_c provide additional biologic information that may be valuable in understanding the variations observed in the natural history of ANLL.

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DEFINING THE PROLIFERATIVE characteristics of malignant cells is a fundamental necessity if one is to understand the natural history of a neoplasm. The leukemias provide an excellent model system for such studies. However, attempts to relate cell cycle characteristics in acute nonlymphocytic leukemia (ANLL) to both the course of the disease as well as the response to therapy have yielded conflicting results.[1-6] This lack of correlation is surprising, since intuitively one would expect that proliferation would be an important determinant of response.

Several potential sources of such inconsistencies are readily identifiable. First, labeling index (LI) studies measuring the percentage of S-phase cells were performed on bone marrow (BM) aspirates in vitro that might yield inaccurate results due to a variable hemodilution of the sample.[7,8] Second, the determination of the durations of S-phase (T_s) and total cell cycle time (T_c) is available on a very small number of patients, the majority of whom had brief life expectancies, so that correlations with the course of the disease could not be sought satisfactorily.[9,10] Most importantly, however, were the problems that existed in the methodology available for such studies. Reviewing these concerns, Perry concluded in 1976[11] that since "the present approaches are either too slow or impractical, new techniques are needed to assess cell cycle times, growth fraction and other important parameters."

We have utilized a new approach by combining immunofluorescence and autoradiography to determine the cell cycle characteristics on 40 leukemic patients.[12,13] This paper describes the relationship of these parameters to the clinical course and the response to therapy in patients with ANLL.

MATERIALS AND METHODS

A total of 40 patients received bromodeoxyuridine (BrdU) infusions; 37 had a diagnosis of ANLL, and the others were in blastic crisis of chronic myeloid leukemia (CML). The BrdU protocol was reviewed and approved by the Roswell Park Memorial Institute's Clinical Investigation Committee as well as the National Cancer Institute (NCI) and the Federal Drug Administration. The BrdU used in this study was supplied by the NCI. Informed consent as per guidelines required by the NCI and FDA was obtained from every patient prior to the administration of BrdU. The methods used in these studies will be described separately in the following sections.

Estimation of the percentage of S-phase cells. After the diagnosis was established, BrdU was administered at a dose of 200 mg/m^2 in 100 mL of 0.9% normal saline. The BrdU was infused intravenously over a period of two hours by using a constant-rate infusion pump in the first 20 patients. The remaining patients received BrdU at 100 mg/m^2 intravenously over a period of one hour. The reason for a decrease in the BrdU dose as well as the infusion time was to infuse the least possible amount of a potential mutagen to patients without jeopardizing results. After the infusion, peripheral blood (PB), BM aspirates, and biopsy samples were obtained from each patient. All samples were handled on ice. Five milliliters of BM was aspirated into a plastic syringe containing 2 mL of 6% sodium citrate. The specimen was layered on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) (specific gravity, 1.077) and centrifuged in a refrigerated centrifuge at 1200 g for 30 minutes. The light-density cells were recovered, washed in RPMI 1640 cell culture medium, and resuspended at a concentration of 1×10^7 cells/mL in RPMI 1640 and 10% fetal calf serum (FCS). All washes were conducted in refrigerated centrifuges, and extreme care was taken to always handle specimens in ice. The samples were then processed by the monoclonal anti-BrdU antibody (RPMB I) as described before.[14,15]

The biopsy samples obtained at the end of BrdU infusion were fixed in Bouin's solution (picric acid, formalin, acetic acid, and water) and processed in plastic by using glycol methacrylate as previously described.[16] Two-micrometer-thick sections of the long-core biopsy samples were obtained by using a tungsten carbide-edged microtome and placed on Alcian blue–coated coverslips. Air-dried sections were then processed by the RPMB technique as described before. After processing, the coverslips were counterstained with hematoxylin and May-Grünwald. At least 2,000 cells

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were counted from each biopsy section from at least five different fields by a single observer. A cell was counted as positively labeled if it showed any brown staining overlying the nucleus. Control samples were treated with only the primary or only the secondary antibody or just 3-amino-9-ethylcarbazole (AEC). There was no positive labeling detected in any of the control samples.

Determination of T_s. T_s was determined in 40 leukemic patients as follows. The post-BrdU infusion BM aspirate was processed in Ficoll-Hypaque as described before. Cells at a concentration of 1×10^7/mL were resuspended in RPMI 1640 and 10% FCS. One hundred microliters of [methyl-^{3}H]-thymidine (stock solution, 20 μCi/mL; specific activity, 60 Ci/mmol; Amersham Corp, Arlington Heights, IL) was added to 1 mL of the cell suspension along with 10 μL of 10^{-4} mol/L fluorodeoxyuridine (FUdR) and incubated for one hour at 37°C in 5% CO_2. After the incubations were completed, cells were washed thrice in cold thymidine and once in phosphate-buffered saline. Three drops of this suspension were placed on Alcian blue–coated coverslips and fixed in 70% ethanol for ten minutes. These coverslips were first processed by the RPMB technique described before and then coated with Nuclear Track Emulsion (NTB_2; Eastman Kodak, Rochester, NY) and exposed in the dark for 48 hours at −70°C. Kodak D19 developer was used to develop the samples, and after the samples were fixed, the coverslips were mounted onto glass slides with Elvanol 51-50 (Dupont) and examined under a fluorescence microscope. At least 2,000 positively labeled cells were counted per slide by a single observer. A cell was considered positively labeled for BrdU incorporation if there was any fluorescence detected over its nucleus and positive for ^{3}H-thymidine (^{3}HTdr) incorporation if at least five or more silver grains were detected on its nuclear surface. The cells considered positive for double labeling demonstrated both fluorescence and silver grains over their nuclei.[17,18] T_s was then calculated by the following formula as described by Wimber and Quastler[19]: $T_s = (DL + {}^3HTdr) \times t/BrdU$, where DL is the number of cells demonstrating double labeling, ^{3}HTdr is the number of cells only showing ^{3}HTdr incorporation, BrdU is the number of cells revealing only BrdU incorporation, and t is the time interval between the two labels. The interval t in patients who received a two-hour BrdU infusion was taken as two hours, and for individuals who received a one-hour infusion of BrdU, it was one hour. The reason we were able to keep this interval constant was because of the extreme care we exercised in obtaining the samples immediately at the end of the BrdU infusion and subsequently handling all samples on ice. Even cell washes were conducted in refrigerated centrifuges so that all metabolic activity in the cells remained arrested until the start of incubation with the second label (^{3}HTdr) at 37°C.

Determination of T_c. Once the values for T_s and the percentage of S-phase cells obtained from the BM biopsy samples were available, the length of T_c was determined by using the formula described before[20] as follows:

$$T_c = T_s \times GF/LI,$$

where GF is the growth fraction and LI is the percentage of S-phase cells as obtained from the biopsy samples. Since we were calculating the cycling times of only those cells that were actively in cycle, the GF was assumed to be 100% in each case.

Clinical data. Prior to administering the BrdU infusion, a diagnosis of acute leukemia was established in every case. Of the 40 cases being reported in this paper, 37 had ANLL with a French-American-British (FAB) staging of M1 through M7, and three patients had blastic crisis of CML. Twenty-six patients had newly diagnosed ANLL, and 11 patients were studied at the time of relapse of ANLL. Of the 26 newly diagnosed ANLL patients, 24 received remission induction therapy, whereas two patients refused chemotherapy and only received supportive care and/or 1 g hydroxyurea orally everyday until the time of death. Twelve patients received remission induction therapy with cytosine arabinoside (araC) at 100 mg/m^2/d by continuous intravenous infusion and adriamycin at 30 mg/m^2/d intravenously for seven (7 + 3) or ten (10 + 3) days plus three days respectively. Twelve patients received high-dose araC at 3.0 g/m^2/d by intravenous infusion over a period of 75 minutes every 12 hours for six days (HDaraC), and one patient also received three days of daunorubicin (DNR) at 45 mg/m^2/d intravenously for the last three days after HDaraC therapy (HDaraC + DNR). Of the 24 newly diagnosed ANLL patients who received the remission induction therapy described earlier, eight entered complete remission (CR), 11 had resistant disease (RD), and five patients died during induction ("other" failures). The treatment failures were classified according to our previously described classification system, which provides for recognizing those individuals who failed therapy because of persistent leukemia (RD) *v* patients who died during induction therapy or during the hypoplastic phase induced by chemotherapy ("others").[21] CR was defined according to the Cancer and Leukemia Group B (CALGB) criteria described before.[22]

All of the 11 ANLL patients studied at the time of relapse received remission induction therapy. Seven were treated with HDaraC, and four received mitoxantrone, 12 mg/m^2/d, intravenously for five days. Two of these latter patients also received 5-azacytidine at 100 mg/m^2/24 hr by continuous intravenous infusion for three days after five days of mitoxantrone therapy. Four patients achieved CR (3/7 CRs in the HDaraC-treated group and 1/4 CR in the mitoxantrone-treated group), five had RD (2/7 in the HDaraC-treated group and 3/4 in the mitoxantrone-treated group), and two patients treated with HDaraC died during induction or were "other" failures.

Control samples. Samples of PB, BM aspirates, and biopsies were obtained from all 40 patients immediately at the end of the BrdU infusion. All samples were placed on ice and transported to the laboratory in the same building. Subsequent cell separation procedures as well as cell washes were conducted in refrigerated centrifuges with cold media. Since minor delays ranging from a few minutes to an hour could in theory occur, we performed the double labeling of samples kept on ice at various times. Immediate post-BrdU infusion BM samples were incubated with ^{3}HTdr, and the cell kinetic data thus obtained were compared with data obtained from the same specimen that was placed on ice for 15, 30, 60, and 120 minutes and then incubated with ^{3}HTdr. Results indicate that no differences were noted for delays of up to two hours as long as the samples were kept on ice. Long delays, however, never occurred in our experience because the protocol was very strictly followed, all specimens being incubated with ^{3}HTdr within 30 minutes.

Statistical methods. Analysis was performed by using the terminal analysis package, a set of interactive computer programs. A two-tailed *t* test was performed to compare the means of the percentage of cells in S-phase from the bone marrow aspirates and the BM biopsy samples. Similarly, a two-tailed *t* test as well as Mann-Whitney and Spearman correlations were performed to compare the T_s, T_c, LI from BM aspirates, LI from BM biopsy samples *v* other clinical or biologic parameters such as the FAB type, height of the WBC count at presentation, cytogenetics, and so on.

RESULTS

Estimation of S-phase cells in vivo. Figure 1 shows an RPMB-processed BM aspirate sample after BrdU infusion in a patient. The use of immunoperoxidase-conjugated secondary antibody and counterstaining permits evaluation of the morphology of the S-phase cells. Using these slides, we were able to assess the percentage of S-phase cells from

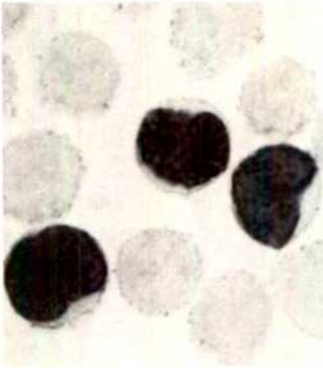

Fig 1. A BM aspirate from a patient with ANLL after a BrdU infusion. The use of immunoperoxidase-conjugated secondary antibody along with the primary monoclonal anti-BrdU antibody and counterstaining permits evaluation of the morphology of S-phase cells.

Table 1. Cell Cycle Studies on Leukemic Patients Following BrdU Infusion

S. No.	% S in Asp	% S in B_x	T_s (h)	T_c (h)
Newly Diagnosed Patients with ANLL				
1	4	16	13	81
2	15	17	25	100
3	16	22	29	132
4	3	12	34	283
5	8	23	21	91
6	2	23	11	48
7	7	25	49	196
8	7	25	4	16
9	6	24	18	75
10	26	50	24	48
11	6	20	13	65
12	6	22	17	77
13	12	14	12	86
14	7	29	18	62
15	3	24	24	100
16	5	23	8	37
17	15	15	9	60
18	11	25	28	112
19	12	21	16	76
20	6	10	23	206
21	16	20	16	80
22	10	23	10	42
23	18	28	9	32
24	8	20	11	52
25	19	22	9	41
26	12	21	13	62
Mean	10%	22%	18	87
Median	8%	22%	16	76
Relapsed ANLL Patients				
27	2	26	18	69
28	7	25	11	44
29	8	15	32	213
30	11	28	14	50
31	17	36	29	81
32	4	23	26	173
33	10	25	33	132
34	2	13	38	292
35	7	18	16	89
36	9	23	9	39
37	12	21	21	100
Mean	8%	23%	22	117
Median	8%	23%	21	89
Blastic Crisis CML Patients				
38	10	30	20	67
39	4	31	13	42
40	8	20	10	50
Mean	7%	27%	14	53
Median	8%	30%	13	50
For All 40 Patients				
Mean	9%	23%	19	93
Median	8%	23%	17	76

Abbreviations: ANLL, acute nonlymphocytic leukemia; CML, chronic myeloid leukemia; S. No., patient no.; % S in Asp, percentage of S-phase cells in bone marrow aspirates; % S in B_x, percentage of S-phase cells in bone marrow biopsies; T_s, duration of S-phase in hours; T_c, duration of total cell cycle time in hours.

matched BM aspirates and plastic-embedded biopsy samples in individual patients. These data are presented for the 40 leukemic patients in the first two columns of Table 1. Clearly, higher LI values were obtained from the biopsy samples (mean, 23%) than from the respective BM aspirates (mean, 9%) in individual patients. The difference in the means of the two groups as determined by the *t* test was statistically significant with a *P* value of .00003. In addition to this striking difference, it is important to note in Table 1 that there is no consistent relationship between the two values in individual patients. For example, patient 1 (Table 1) had 4% S-phase cells in the aspirate and 16% in the biopsy sample, whereas patient 2 had a LI of 15% in the aspirate and 17% in the biopsy sample.

Measurement of cell cycle times. T_s as well as T_c were determined on 40 leukemic patients. Table 1 provides this information in detail. Thirty-seven patients had a diagnosis of ANLL, 26 having been studied at the time of initial diagnosis and 11 at the time of relapse. Three patients were studied in blastic crisis of CML. If all the leukemic patients studied are considered together, one can see from Table 1 that there are wide variations in both T_s as well as T_c between individuals, although the mean and median values for each correspond well with what has been reported in the literature (mean T_s, 19 hours; median, 17 hours; and mean T_c, 93 hours; median 76 hours). The T_s ranges from four to 49 hours,and the T_c ranges from 16 to 292 hours. Table 1 also compares the cell cycle characteristics of newly diagnosed and relapsed ANLL patients as well as three individuals studied during blastic crisis of CML. These last three patients demonstrate shorter T_s and T_c as compared with the ANLL group. Although not statistically significant because of the few patients studied, it appears that both T_s and T_c of the leukemic cells in relapsed individuals are longer than that for newly diagnosed ANLL (mean of 22 hours *v* 18 hours for T_s and mean of 117 hours *v* 87 hours for T_c, respectively).

Since T_c is a derivative of both T_s and LI, we determined its relationship to these two parameters separately. Although there was no correlation between T_c and LI ($r = .4$), there appeared to be a statistically significant correlation ($r = .8$)

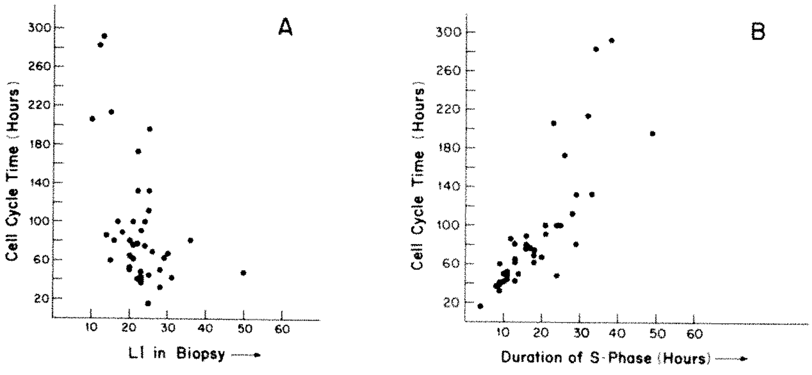

Fig 2. Relationship of T_c to the percentage of S-phase cells (A) and T_s (B) was determined in patients with ANLL. Although the correlation for the former was low ($r = .4$), there was a statistically significant relationship between T_c and T_s ($r = .8$).

between T_c and T_s. Figures 2A and 2B show these comparisons. We also assessed the relationship between T_s and T_c and the FAB type, the cytogenetic karotype, and the height of the WBC count at presentation. There was no unique relationship between any of these values. For example, in nine newly diagnosed ANLL patients with FAB-M2, the median value for T_s was 12 hours, and that for T_c was 72 hours. Similarly, in ten newly diagnosed ANLL patients with FAB-M4, the median values for T_s and T_c were 15 and 70 hours, respectively.

Relationship of cell cycle characteristics to clinical course of the disease in ANLL. Although the means of T_s and T_c for all leukemic patients studied approximated closely with what has so far been reported in the literature, it is clear that there are distinct differences between individual patients. For example, the leukemic cells of seven patients had T_c values that differed from the population mean value by more than 1 SD (ie, either less than 23 hours or greater than 157 hours). All seven patients (Table 1, patient numbers 4, 7, 8, 20, 29, 32, and 34) failed to enter CR after chemotherapy, six because of persistent leukemia after therapy and one because of early death. Five of the six treatment failures had a very long T_c (173, 196, 203, 213, 282 and 292 hours, Table 1), whereas one had a very short T_c (16 hours). Although chemotherapy produced marrow aplasia in the latter patient, leukemic cells rapidly repopulated the marrow.

The values of T_s and T_c for patients studied at the time of initial diagnosis of ANLL *v* the outcome of remission induction therapy are presented in Table 2. It appears at a glance that both T_s and T_c are shorter (mean T_s, 16 hours; mean T_c, 66 hours) in patients who achieved CR as compared with those who had RD (mean T_s, 21 hours; mean T_c, 104 hours). Of note in the RD group is that two patients had a very long T_c (196 and 283 hours), whereas two patients had a very short T_c (16 and 38 hours), which suggests a possible role of cell cycle-related "kinetic resistance" to therapy in these individuals.

DISCUSSION

The proliferative characteristics of malignant cells were studied in 40 leukemic patients. The data presented in this paper led to three important observations. First, the percentage of S-phase cells was much higher in BM biopsy specimens than in simultaneously obtained BM aspirates. Second, there was great variability in cell cycle times of individual patients ranging from very short to very long durations of both the S-phase and the cycling times of cells. Finally, there was no direct relationship between the length of the cell cycle and the percentage of cells actively synthesizing DNA.

In previous studies, we have demonstrated that the in vitro BrdU LI were similar to the in vitro ^{3}HTdr LI.[23] In the present study, the in vivo BrdU labeling of BM aspirates provide data comparable with studies that have used ^{3}HTdr in vivo. For example, the mean LI in BM aspirates after a BrdU infusion in this study is 9% compared with the mean LI after a ^{3}HTdr infusion reported in the literature ranging between 5.9% to 7%.[24-28] Similarly, the observation of a higher LI from matched BM biopsy samples of these patients confirms the findings of previous investigators[7,8,29] who have addressed the question of hemodilution of the aspirates.

Many attempts have been made to relate the proliferative characteristics of leukemic cells as measured by the LI of BM aspirates with treatment outcome.[30-32] The results have been conflicting. Obviously, one reason for this lack of relationship may be that the value for the percentage of S-phase cells was obtained from BM aspirates, which as noted earlier may provide inaccurate information. Even if

Table 2. Cell Cycle Studies on Newly Diagnosed Patients With Acute Nonlymphocytic Leukemia Who Received Remission Induction Therapy *v* Outcome of Treatment

CR			RD			Others		
S. No.	T_s (h)	T_c (h)	S. No.	T_s (h)	T_c (h)	S. No.	T_s (h)	T_c (h)
3	29	132	2	25	100	1	13	81
9	18	75	4	34	283	6	11	48
10	24	48	5	21	91	17	9	60
19	16	76	7	49	196	20	23	206
21	16	80	8	4	16	24	11	52
22	10	42	13	12	86			
23	9	32	14	18	62			
25	9	41	15	24	100			
			16	8	37			
			18	28	112			
			26	13	62			
Mean	16	66		21	104		13	89
Median	16	61		21	92		11	60

Abbreviations: CR, complete remission; RD, resistant disease; Others, death during induction; S. No., patient no.; T_s, duration of S-phase in hours; T_c, duration of total cell cycle time in hours.

the LI were accurate, it is not the only determinant of the biologic behavior of leukemic cells since it provides no information about T_s or T_c, which are far more important measurements than the LI alone. The most striking illustration of this can be observed in Table 1, where both patients 7 and 8 have 7% S-phase cells in the BM aspirate and 25% S-phase cells in their biopsy specimens, yet their T_s (49 *v* 4 hours) and T_c (196 *v* 16 hours) are entirely unrelated. One patient (no. 7) takes approximately eight days to double his leukemic burden, whereas the other (no. 8) is doubling his leukemic cell mass every 16 hours. Hence, in order to acquire a comprehensive view of the cell cycle characteristics in ANLL patients, all three characteristics, ie, LI as well as T_s and T_c, must be taken into account.

Futhermore, conclusions regarding cell cycle characteristics in newly diagnosed ANLL patients have in the past been based on approximately 20 patients from nine different studies.[25,27,28,33-38] Because the dose level and schedule of ^{3}HTdr infusions as well as the methodology of sample processing was quite different in each study, comparisons of such data are difficult. Since the methodology we used is considerably more efficient than the double-labeled autoradiography or the estimation of labeled mitosis used in previous studies, we were able to study large numbers of patients uniformly.

After administration of BrdU and in vitro incubation of BM aspirates with ^{3}HTdr, we used our previously described double-label technique to measure the T_s and T_c in 40 leukemic patients. The internal t between the two labels was maintained at a constant by obtaining the BM samples immediately at the end of the BrdU infusion and subsequently handling samples on ice. All cell washing procedures were conducted with cold media in refrigerated centrifuges so that metabolic activity in the cells remained arrested until the start of incubation with the second label (^{3}HTdr) in vitro at 37°C.

Cell cycle characteristics were thus measured on 40 leukemic patients, 26 of whom were studied at the time of initial diagnosis of ANLL, 11 at the diagnosis of relapsed leukemia, and three individuals in blastic crisis of CML. Of the 26 newly diagnosed ANLL cases studied, 24 subsequently received remission induction therapy. It appears that 11 patients who survived therapy (CR + RD only) had a T_c of less than 80 hours, and seven of these 11 patients achieved CR (Table 2). On the other hand, among eight patients who had a T_c longer than 80 hours and who survived therapy (CR + RD), only one entered remission, and seven demonstrated clinical resistance. This seems to indicate that patients with a T_c longer than 80 hours tend to respond unfavorably to chemotherapy. In fact, of six ANLL patients who had an extraordinarily long T_c (173, 196, 203, 213, 282, and 292 hours, Table 2), five demonstrated resistance to chemotherapy, and one patient died during treatment.

On the other hand, patients at the other extreme of T_c do not respond well either. For example, the patient with T_c of 16 hours (no. 8, Table 1) achieved aplasia after chemotherapy, but his BM was repopulated by rapidly dividing leukemic cells. This patient is particularly interesting because he achieved aplasia twice after chemotherapy, and each time his BM was repopulated by leukemic cells. The regrowth rate of his leukemic cells as measured by the daily increase in myeloblasts from his PB both times was consistent with a T_c ranging between 19 to 21 hours (manuscript in press). This value correlated strongly with a cycling time of 16 hours for the myeloblasts we had determined prior to administration of any chemotherapy. It must be remembered that for the calculation of T_c we utilized the LI value from biopsy samples rather than BM aspirates. Had we used the LI value from the BM aspirate in this patient, the calculated T_c would have been 57 hours rather than 16 hours. Since the clinical and biologic behavior of his leukemic cells was more consistent with a cycling time of 16 hours, this case validates the use of biopsy specimens for a more accurate assessment of the percentage of cells actively engaged in DNA synthesis.

Table 1 also compares the cell cycle characteristics of newly diagnosed and relapsed ANLL patients as well as three individuals studied during blastic crisis of CML. The three CML patients demonstrate a shorter T_s and T_c compared with the ANLL group, an observation consistent with the fact that, although chemotherapy often produces marrow aplasia, rapid regrowth of blastic crisis clones often occurs. Similar to previously reported results,[39] both the T_s and T_c in relapsed ANLL patients were longer than that for newly diagnosed ANLL (22 *v* 18 hours for T_s and 177 *v* 87 hours for T_c, respectively). Therefore, selection of leukemic clones that are slowly cycling may be an additional explanation for the frequent resistance to chemotherapy observed in these patients.

It must be remembered, however, that the biologic properties of leukemic cells represent only one of several factors that help to determine response. It is only possible to say that some cell cycle characteristics may be identified that if present will result in an unfavorable response to therapy, but if absent does not guarantee CR. Therefore we may see patients who have cell cycle characteristics compatible with a favorable response to chemotherapy yet who fail to enter remission because of other reasons (such as metabolic drug resistance of leukemic cells or a very high leukemic cell mass at the time of treatment). The clinical implications of identifying a subset of patients who will manifest resistance to therapy either because of very slowly cycling cells or unusually rapid proliferation regardless of other favorable prognostic factors are then obvious. For example, if this information can be obtained prior to therapy, both remission induction as well as consolidation/maintenance regimens could be altered to suit the individual needs of patients. Thus patients with a long T_c may benefit from prolonged treatment utilizing low doses of drugs. On the other hand, individuals with very rapidly dividing leukemias may be candidates for intermittent therapy with intensive courses of S-phase–specific agents. Finally, the duration of remission in patients who achieve CR may at least in part depend upon the regrowth rate of the residual leukemic cells; however, the present study is too young for comment on this aspect.

One last concern that needs to be addressed in the assessment of cell cycle characteristics is the question of the actual growth fraction in each patient. We assumed it to be 100% in the calculations of T_c. This assumption was based on the observations reported in three elegant studies[37,40,41] that have shown that the noncycling or G_0 compartment of cells in ANLL is relatively small. For example, Clarkson et al[37] administered ^{3}HTdr by continuous infusion for eight to ten days and demonstrated that 90% to 98% BM cells were labeled at the end of this period. This left a definite but insignificant proportion of 2% to 8% noncycling cells in the G_0 compartment. Although it would be ideal to measure even this small subcompartment in every patient, at the present time the risk of administering BrdU by continuous infusion for long periods of time is not justified. Second, it must be remembered that the methods we utilized in our studies as well as those in the past can only yield information about the average behavior of a cell population. No information can be obtained about the rate of DNA synthesis or duration of cell cycle time in individual cells. It is possible that the 2% to 8% unlabeled cells in the study of Clarkson et al were very slowly cycling rather than being truly quiescent. Therefore, similar to past experience and assuming all cells to be in cycle, we have estimated the average cell cycle times of leukemic cells in a population.

In summary, therefore, cell cycle characteristics were determined for 40 leukemic patients. There were distinct differences in both the T_s as well as the T_c of individual patients. A subgroup of individuals was identified with either a very long or very short T_c who demonstrated clinical drug resistance in response to multiple courses of chemotherapy. Because these measurements can now be obtained within a reasonable time frame, they will contribute greatly in understanding the biologic behavior and the natural history of acute leukemias as well as assist in tailoring therapy to suit the needs of individual patients.

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Hematologic Effects of Immunotherapy With Lymphokine-Activated Killer Cells and Recombinant Interleukin-2 in Cancer Patients

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Immunotherapy with interleukin-2 (IL-2) and lymphokine-activated killer (LAK) cells generated from autologous lymphocytes has produced significant tumor regressions in patients with advanced cancer. In the current study, we reviewed the hematologic effects associated with this therapy in our initial 42 patients. Eighty-eight percent of the treated patients developed anemia that required $\geq$4 units of red cell transfusions, and 43% received at least 8 units. Only a blood loss of 2 to 3 units could be attributed to repeated phlebotomy, cytophereses, and hemodilution. IL-2 administration also resulted in thrombocytopenia as well as lymphopenia and eosinophilia. Forty-three percent of patients developed platelet counts of $\leq$50,000/μL, and 36% of the total group required platelet transfusions. Mild neutropenia and a rebound lymphocytosis followed discontinuation of IL-2 treatment. To explore the possible mechanisms for these hematologic effects, standard hematopoietic colony assays were conducted on serial blood samples from five patients. IL-2 produced a significant decline in circulating erythroid (BFU-E) and granulocytic/macrophage (CFU-C) progenitors, which rebounded after the discontinuation of IL-2 therapy. Infusion of IL-2 also resulted in measurable serum levels of γ-interferon. Some of the hematologic effects of immunotherapy with LAK cells and IL-2 may be the result of IL-2–mediated suppression of hematopoiesis.

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WE HAVE RECENTLY reported that the intravenous administration of lymphokine-activated killer (LAK) cells and recombinant human interleukin-2 (IL-2) to humans with disseminated cancer can produce significant regressions of measurable tumor.[1,2] Autologous lymphocytes, which are harvested by repeated leukophereses, are incubated for three to four days in IL-2 to generate LAK cells. These cells are capable of lysing fresh, natural killer-resistant tumor cell targets but not normal cells in short-term chromium release assays.[2-4] Although promising as an anticancer treatment,[1,2] this immunotherapy has been associated with multiple adverse effects including fever, chills, malaise, confusion, diarrhea, and a poorly defined vascular leak syndrome.[1,2,5] Furthermore, 24 of the initial 25 patients treated required red cell transfusions for anemia, and thrombocytopenia (nadir platelet count, $\leq$50,000/μL) was observed in 44% of the patients.[1,2]

In the current investigation we conducted a detailed survey of the hematologic effects observed in our initial 42 immunotherapy patients. To study the possible pathogenesis of some of the observed hematologic toxicities, we also investigated the in vivo effects of this immunotherapy upon circulating erythroid (BFU-E) and granulocytic/macrophage (CFU-C) progenitor cells in five patients.

MATERIALS AND METHODS

Patients and treatment. The analysis of the hematologic effects of immunotherapy with LAK cells and IL-2 was drawn from the clinical summaries of our initial 42 patients undergoing their first round of treatment. Serial studies of circulating hematopoietic progenitors were conducted on four patients receiving LAK cells and IL-2 and one patient undergoing therapy with IL-2 alone. The recombinant IL-2 used in this trial was kindly supplied by Cetus Corp (Emeryville, CA). The molecular biologic and immunologic properties of this lymphokine preparation have been described elsewhere.[6] Summaries of the clinical histories (Table 1) and of hematopoietic colony assay results (Table 2) are presented for the five patients who underwent analysis of the IL-2 effect upon peripheral blood progenitor colonies.

All patients had advanced cancer and had failed effective standard therapy for their specific neoplasm. Prior to the initiation of treatment, none of the patients had significant, nonneoplastic hematologic, cardiopulmonary, renal, or hepatic disease. No patient had received any therapy for at least 4 weeks before entry into this treatment trial. Informed consent was obtained from all patients before beginning therapy. The experimental immunotherapy protocol was previously approved by the Clinical Research Committee of the National Cancer Institute and by the Food and Drug Administration.

The regimen for leukophereses and lymphocyte harvest and culture has been previously described in detail.[1] Briefly, repeated lymphocytophereses using a continuous-flow, cell separator (IBM-2997; Cope Labs, Lakewood, CO) were performed to collect 5×10^9 to 3×10^{10} mononuclear cells per procedure. Lymphocytes were separated by centrifugation over Ficoll-Hypaque density gradients (LSM; Litton Bionetics, Rockville, MD) and were then placed in human AB serum–containing medium. After incubation at 37°C in roller bottles for three to four days, the LAK cells were harvested by centrifugation and were resuspended in 0.9% saline containing human serum albumin and IL-2 (375 U/mL). The cell suspension was filtered through sterile nylon mesh before infusion.

The treatment protocol has been detailed previously.[1] Briefly, patients received three to four days of 30,000 or 100,000 U/kg of IL-2 administered as bolus intravenous (IV) infusions every eight hours. After a two- to three-day period not receiving IL-2, daily leukophereses were performed for the next five consecutive days. This schedule for the leukophereses was chosen to maximize lymphocyte yields by taking advantage of the lymphocytosis that follows the discontinuation of IL-2 infusion.[1,7] The LAK cells were then administered by the IV route three to four days later. After the first cell infusion, 10,000 to 100,000 U/kg of IL-2 were injected IV every eight hours and continued for an average of four to five days. Discontinuation of IL-2 therapy was dictated by the development of

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Table 1. Patient Population

Patient	Age	Sex	Primary Malignancy	Sites of Metastases	Prior Therapy
1	42	F	Adenocarcinoma of the breast	Subcutaneous tissues, liver, lungs	Surgery, radiation (axilla, supraclavicular fossa)
2	63	F	Melanoma (back)	Lung, pancreas	Surgery
3	33	M	Alveolar rhabdomyosarcoma (thigh)	Lung	Surgery, chemotherapy (cyclophosphamide, doxorubicin, methotrexate, vincristine, VP-16, cisplatin, actinomycin D dacarbazine), radiation (thigh, lungs)
4	52	F	Adenocarcinoma of the lung	Mediastinal lymph nodes, lungs	Radiation (mediastinum)
5	45	M	Clear cell carcinoma of the kidney	Lungs, kidneys	Surgery

toxicity. After a rest period, the cycle of leukophereses and LAK cell infusions with IL-2 was repeated in some patients. Some patients were treated with high-dose IL-2 alone and, therefore, did not undergo repeated leukophereses. The therapy was administered in two four- to six-day cycles of 100,000 to 300,000 U/kg of IL-2 given every eight hours.

During the intervals of IL-2 administration, patients received acetaminophen, indomethacin, ranitidine, hydroxyzine, doxepin, and meperidine.

Standard blood studies including complete blood counts, hepatic and renal function tests, and electrolyte determinations were frequently performed during the treatments.

Hematopoietic colony assays. The effect of immunotherapy on hematopoietic colony formation in vitro[8] was measured before, during, and after administration of IL-2. Peripheral blood was collected in 50 units of preservative-free heparin (Forest Pharmaceuticals, St Louis) per milliliter of blood. Mononuclear cells were separated by sedimentation over Ficoll-Hypaque (LSM) and washed twice in Iscove's modification of Dulbecco's medium (GIBCO, Grand Island, NY) with 2% heat-inactivated fetal calf serum (FCS, Flow Laboratories, McLean, VA). The cells were then resuspended in culture medium to a final concentration of 3×10^5 cells/mL. Culture medium contained 0.8% methylcellulose (Dow Chemicals, Midland, MI), 30% FCS, 1% bovine serum albumin (Sigma Chemical Co, St Louis), 100 U/mL penicillin-streptomycin (M.A. Bioproducts, Walkersville, MD), 2.5 U/mL of erythropoietin (Connaught Laboratories, Willowdale, Canada), and 10% phytohemagglutinin (GIBCO)-stimulated leukocyte-conditioned media. Cultures were plated in duplicate in Lux suspension culture dishes (35 × 10 mm; Miles Scientific, Naperville, IL) and incubated at 37°C in 95% humidity and 5% CO_2. Colonies derived from primitive erythroid (BFU-E) and granulocyte/macrophage (CFU-C) progenitors were enumerated by their characteristic morphologies on day 18. The data are expressed as the number (mean ± SD) of colonies per milliliter of blood to correct for both changing total blood counts and variable recovery of mononuclear cells during treatment.

Table 2. Summary of Hematopoietic Progenitor Colonies Before (Pre), During, and After (Post) IL-2 Therapy

	BFU-E*			CFU-C*		
Patient	Pre	During†	Post‡	Pre	During†	Post‡
1	31	2	76	10	0	7
2	124	1	27	3	0	19
3	64	2	22	4	0	15
4	0	0	2,870	0	0	462
5	20	0	78	6	0	29

*Values are colonies per milliliter of blood; SD, <15% of reported values.

†Minimum colony number during IL-2 treatment.

‡Maximum colony number after IL-2 treatment.

Interferon assay. γ-Interferon levels were determined with a commercial radioimmunoassay kit (Centocor, Philadelphia) using a National Institutes of Health γ-interferon standard (Gg 23-901-530) as an internal control.

RESULTS

The hematologic profiles for the initial 42 patients receiving LAK cells and IL-2 are presented in Table 3. Figure 1

Table 3. Hematologic Effects of First Treatment Round With LAK Cells and Recombinant IL-2 in Initial 42 Patients

	Patients		Cell Counts (× 10^3/μL Blood)		
	Cell Count (× 10^3/μL)	Number (%)	Mean	Median	Range
Nadir values					
Platelets	≤80	27 (64)	67.62	55.00	7.0-186.0
	≤50	18 (43)			
	≤20	9 (21)			
Lymphocytes	≤1	41 (98)	0.14	0.05	0.0-1.7
	≤0.2	34 (81)			
	0	19 (45)			
Neutrophils	≤2	11 (26)	2.64	2.49	0.3-5.6
	≤1	3 (7)			
	≤0.5	1 (2)			
Total leukocytes	≤4	29 (69)	3.28	3.15	0.7-6.5
	≤2.5	15 (36)			
	≤1	3 (7)			
Peak values					
Eosinophils	≥10	5 (12)	5.91	2.96	0.4-37.0
	≥5	16 (38)			
	≥2	28 (67)			
Lymphocytes	≥15	7 (17)	10.09	7.09	2.4-43.5
	≥10	13 (31)			
	≥5	30 (71)			

Normal values: total leukocytes, 5,000 to 11,000/μL; neutrophils, 2,500 to 7,700/μL; eosinophils, 140 to 500/μL; lymphocytes, 1,000 to 4,000/μL; platelets, 145,000 to 450,000/μL.

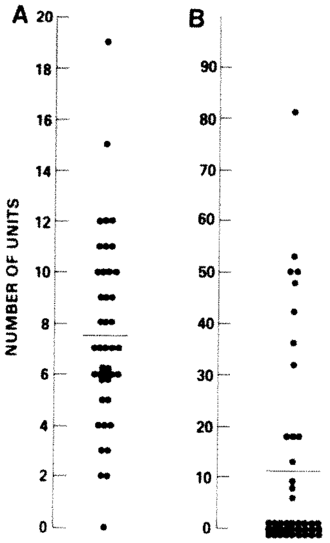

Fig 1. Packed red cell (A) and platelet (B) transfusion requirements of the initial 42 patients receiving immunotherapy with LAK cells and IL-2. Each dot represents a single patient. The mean (horizontal bars) and median numbers of red cell transfusions were 7.5 and 7 units, and those of platelet transfusions were 11.3 and 0 units, respectively.

shows the transfusion requirements resulting from therapy for this same group of patients.

IL-2 infusions resulted in moderate to severe thrombocytopenia (mean and median nadir counts, 67,620 and 55,000/μL, respectively), with 64% of the patients having platelet counts $\leq$80,000/μL and 21% having counts $\leq$20,000/μL. One patient whose platelet count fell as low as 20,000/μL developed an upper gastrointestinal hemorrhage, but no other significant bleeding episodes occurred as a result of thrombocytopenia.

The administration of IL-2 led to a marked lymphopenia (mean and median nadir counts, 140 and 50/μL, respectively), with lymphocyte counts falling below 1,000/μL in 98% of patients and to 0 in 45% of the individuals. After discontinuation of IL-2 therapy, a rebound lymphocytosis, which has been reported previously,[7] was observed (mean and median peak counts, 10,094 and 7,090/μL, respectively).

Eosinophilia (mean and median peak counts, 5,913 and 2,963/μL, respectively) also developed during the course of immunotherapy and was usually observed at the end of treatment or during the five days after completion of therapy. Sixty-seven percent of the individuals had peak eosinophil counts $\geq$2,000/μL, whereas 38% demonstrated counts $\geq$5,000/μL. Although IL-2 did induce generalized fluid retention and a pruritic dermatitis, no allergic phenomena such as urticaria or bronchospasm were associated with the eosinophilia of IL-2 therapy.

Moderate leukopenia (mean and median nadir counts, 3,280 and 3,150/μL, respectively) and, in some patients, mild neutropenia (mean and median nadir counts, 2,640 and 2,490/μL, respectively) were noted during therapy. In general, leukopenia was associated with the lymphopenic episodes that occurred during IL-2 infusion and not with neutropenia. Only three patients developed neutrophil counts of $\leq$1,000/μL, and none experienced septic complications.

The severity of the hematologic toxicity was reflected by extensive transfusion requirements (Fig 1). Forty-one of the 42 patients required transfusions of packed red cells, platelets, or both during therapy. Although no patient had received any other cancer treatment within 30 days of beginning immunotherapy, 14 patients had at some prior date received chemotherapy or immunotherapy, five individuals had previously undergone radiation treatments, and seven patients had received both. Red cell transfusions were administered during therapy to maintain the hemoglobin at $\geq$10 g/dL. Transfusions of 4 units of packed red cells were necessary in 37 patients (88%) and 8 or more units in 18 individuals (43%). Platelet transfusions were administered for bleeding in the setting of thrombocytopenia or for prophylaxis when platelet counts fell below 20,000/μL. Although 64% of patients never required platelet transfusions, eight individuals (19%) received over 20 units. One patient did not require any transfusions, whereas another patient, who suffered a gastrointestinal bleed (see the second paragraph of Results) during therapy-induced thrombocytopenia, required 19 units of packed red cells and 48 units of platelets. Except for six other patients with occult blood loss from the gastrointestinal tract and one with minor episodes of hemoptysis, there was no other evidence for significant intestinal, respiratory, or urinary tract blood losses or hemolysis. Phlebotomy during the treatment period accounted for the loss of approximately 1 to 1½ units of blood per patient. Hemodilution due to the administration of crystalloid and colloid solutions also contributed to changes in the concentration of various blood elements. No evidence of autoimmune disease was detected in the patients during or after therapy.

The clinical courses of the following two patients are representative of our findings. Patient 1 (Fig 2) received the combination therapy with LAK cells and IL-2, whereas patient 2 (Fig 3) was treated by high-dose IL-2 alone and did not undergo multiple leukophereses and LAK cell infusions.

With the initial administration of IL-2 (30,000 to 300,000 U/kg IV every eight hours), a significant fall in the hemoglobin level and suppression of BFU-E–derived colonies were seen when IL-2 was administered (Figs 2 and 3, Table 2). In patient 1, IL-2 produced a five- to 15-fold reduction in BFU-E colony numbers below the immediate pretreatment baselines, and in patient 2 the therapy resulted in a five- to 125-fold decrease in erythroid colonies from the baseline. Since the reduction of hemoglobin and circulating erythroid progenitor levels was observed with or without concurrent LAK cell administration, this effect appeared to represent an IL-2–mediated, LAK cell–independent phenomenon. That bone marrow function was affected by IL-2 therapy could also be inferred from the drastic decrease in colony numbers and reticulocytopenia (see patient 1, Fig 2). Although the suppression by IL-2 was evident upon both BFU-E and CFU-C colonies, the effect was much more striking upon the erythroid progenitors (Table 2). In both patients, the treatment-induced anemia necessitated 6 units of red cell transfusions. Patient 2 required 37 units of platelet concentrates during periods of severe thrombocytopenia.

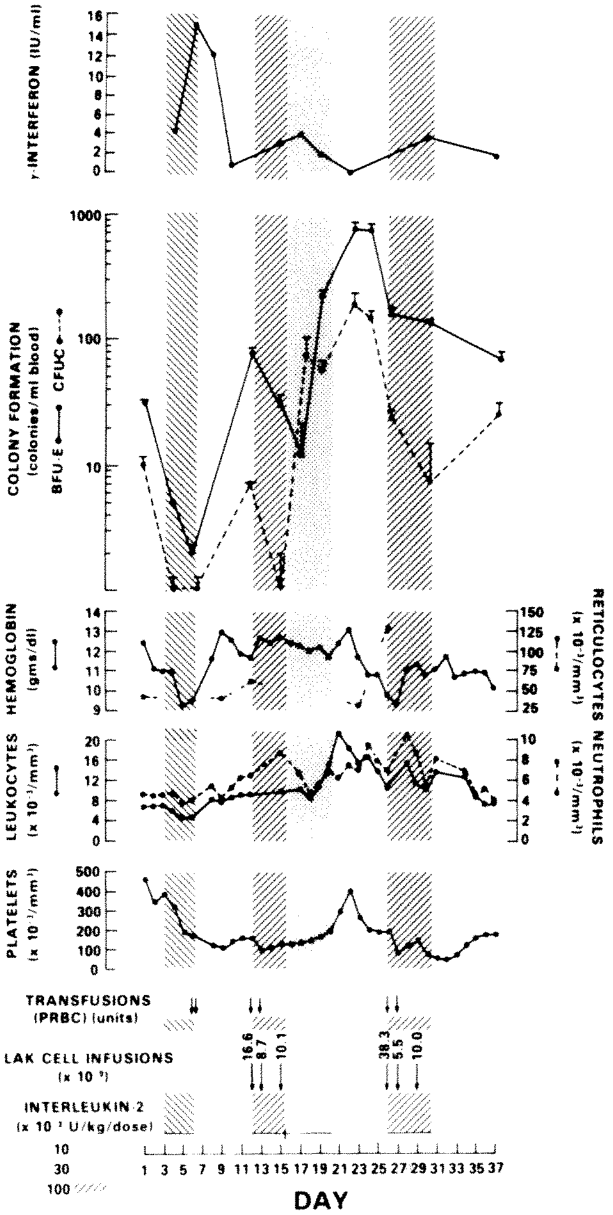

Fig 2. Hematologic effects of LAK cells and IL-2, patient 1. Serial measurements of γ-interferon, erythroid (BFU-E) and granulocytic/macrophage (CFU-C) progenitors (mean ± SD), hemoglobin, reticulocyte counts, total leukocytes, neutrophils, platelets and packed red cell (PRBC) transfusions are shown. LAK cell infusions were administered as shown after leukophereses on days 8 to 12 and 22 to 26, inclusively. IL-2 was given IV every eight hours at the doses indicated.

After the withdrawal of IL-2 therapy the number of circulating erythroid precursors rebounded dramatically above the baseline (Figs 2 and 3, Table 2). The extent of this increase appeared to be directly related to the dose of IL-2 administered. For example, in patient 1 (Fig 2), the erythroid colony number rose 38-fold above the nadir level after 30,000 U/kg of IL-2 and 64-fold after 100,000 U/kg.

Circulating γ-interferon could be measured during periods of IL-2 administration. For example, in patient 1 (Fig 2), the highest level of interferon (15.0 IU/mL on day 6) was observed with the initial injections of IL-2 at 30,000 U/kg/dose. However, there did not appear to be a direct dose correlation between injected IL-2 and measured interferon, since the later doses of IL-2 (100,000 U/kg/dose) did not result in higher levels of interferon. Similarly, in patient 2, interferon levels rose to 8.5 IU/mL on day 9 (after four days of IL-2 given every eight hours) and declined to 0 by four and 60 days after completing IL-2 therapy.

DISCUSSION

Recently, the administration of LAK cells and recombinant IL-2 to humans with advanced cancer has produced a reduction in measurable tumor in 11 of 25 patients.[1,2] However, this therapeutic regimen has been accompanied by significant toxicity. We report in this study major hematologic changes in the initial 42 patients undergoing their first cycle of immunotherapy. Anemia that required red cell transfusions occurred in almost every patient treated with LAK cells and IL-2. Thrombocytopenia (platelet count, ≤50,000/μL) was observed in 43% of the individuals, and 36% of the total patient group received platelet transfusions. Sixty-nine percent of those treated developed total leukocyte counts under 4,000/μL, although severe neutropenia (≤500/μL) was only observed in one individual. Other hematologic effects included lymphopenia and eosinophilia. Since large human trials with LAK cells and IL-2 are currently under way, the knowledge of these hematologic effects and an understanding of their pathophysiology assume particular importance.

The rapidity of the decline in hemoglobin and platelet levels during therapy with IL-2 could be partly attributed to phlebotomy, hemodilution, and in some patients, gastrointestinal tract losses. In addition, in those individuals receiving LAK cells and IL-2, repeated cytophereses also resulted in small blood losses, since each cytopheresis procedure generated 350 to 550 mL of a cell suspension with a hematocrit value of 3% to 5% and 1 to 9 × 10^{11} platelets. However, even these processes taken collectively could not account for the severity of the anemia and the large number of transfusions required by these patients. Blunting of the normal hematopoietic response to ongoing minor blood losses appeared likely.

We investigated the possible mechanism underlying the anemia, leukopenia, and thrombocytopenia induced by IL-2 therapy by using clonal assays for hematopoietic progenitors. Serial colony numbers were determined by using peripheral blood rather than bone marrow for practical and ethical reasons in critically ill patients with many acute medical problems. Because observations had revealed that hematologic abnormalities returned to normal in all patients after ceasing IL-2 administration, we felt that repeated bone marrow aspirations were not justified in this patient population. In general, the peripheral blood colony number reflects hematopoiesis in hypoproliferative and hyperproliferative hematologic diseases such as aplastic anemia[9] and chronic myelogenous leukemia[10] or polycythemia vera.[11] That the striking and rapid decline in the peripheral blood colony number was significant in the present study was suggested by (1) the concurrent and consistent fall in the patients' blood counts and (2) the regular rebound to relatively high colony

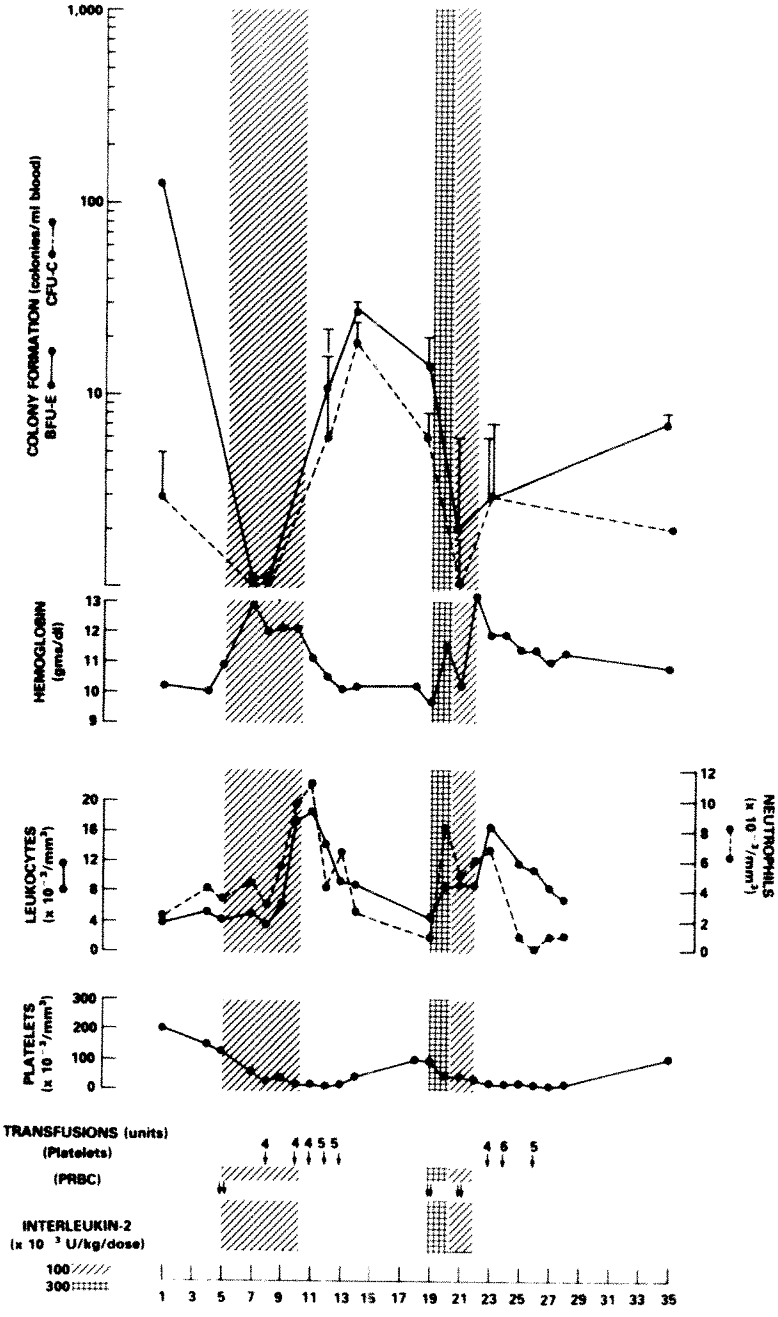

Fig 3. Hematologic effects of IL-2, patient 2. (See the legend for Fig 2.) This patient received only high-dose IL-2 and therefore did not undergo repeated leukophereses or LAK cell infusions.

numbers with the termination of IL-2 infusions. In the only patient examined by bone marrow aspirate and biopsy during IL-2 therapy, there was absence of bone marrow precursor cells coincident with the low number of hematopoietic colonies in peripheral blood.

The hematologic changes were almost certainly secondary to IL-2 administration. The three- to 125-fold decline in erythroid colony number in the circulation of treated patients occurred with the administration of IL-2 alone as well as with the combination of LAK cells and IL-2. Moreover, these falls in colony numbers preceded the period of intensive cytophereses in patients receiving both LAK cells and IL-2. It is unlikely that LAK cells produced significant lysis of hematopoietic colonies since LAK cells have not demonstrated the ability to destroy normal tissues[2,12] despite their broad lytic reactivity against a wide variety of tumor types.[1-4]

Both negative[13,14] and positive[15,16] effects of IL-2 on hematopoiesis have been reported in colony culture. Recombinant IL-2 has been shown to inhibit hematopoiesis in vitro in a dose-dependent manner, with the effect mediated by Tac+[13] or OKT8+[14] cells. At low concentrations[15] or under special culture conditions,[16] recombinant IL-2 has also demonstrated stimulatory activity upon colony growth. These discrepant results almost certainly are due to variability among clonal progenitor assay models and the complex interactions between IL-2 and the repertoire of immune cells that regulate hematopoiesis in vitro.

IL-2 also has profound effects upon the number of peripheral blood lymphocytes in both animals and humans; the administration of IL-2 results in a dramatic fall in all subpopulations of lymphocytes,[7] possibly due to a redistribution into nonrecirculating compartments.[17] Continuous infusion of IL-2 to humans leads to an eventual rebound in all T cell subsets, including cytotoxic/suppressor cells, and the appearance of cells bearing the Tac antigen,[7] a marker of lymphocyte activation.[18,19]

IL-2 can induce the proliferation of lymphocytes in vitro[20] and in vivo[17] as well as stimulate cultured lymphocytes to produce lymphokines with a variety of regulatory effects.[21-23] The addition of IL-2 to lymphocyte cultures can result in the induction of suppressor cells.[24,25] The activity of these stimulated lymphocytes and soluble factors may, in turn, be modulated by the presence of other cells and cytokines. Serum samples from patients receiving LAK cells and IL-2 contained significant quantities of γ-interferon, an expected result given the ability of IL-2 to induce γ-interferon production in stimulated cultures.[23] The interferons are potent suppressors of hematopoietic cell proliferation in vitro.[26-29] In addition, γ-interferon may mediate suppression, is synergistic with other inhibitory cytokines, as well as stimulate the activity of a wide range of suppressive cell types.[29,30] The pattern of hematopoietic suppression in these treated cancer patients is similar to the constellation of findings in individuals with aplastic anemia; in both groups, low numbers of progenitor colonies are associated with high levels of IL-2 and γ-interferon and lymphocyte activation.[31,32]

Although the pathogenesis of IL-2–induced depression of hematopoietic proliferation remains unclear, it appears likely that IL-2 has both stimulatory and indirect inhibitory effects upon many subpopulations of cells within the lymphoid cell repertoire. The suppression of hematopoiesis mediated by the administration of IL-2 probably represents a complex in vivo interplay of several regulatory lymphokines.

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Development of Large Numbers of Mast Cells at Sites of Idiopathic Chronic Dermatitis in Genetically Mast Cell–Deficient WBB6F$_1$-W/W^v Mice

By Stephen J. Galli, Naoki Arizono, Tatsuo Murakami, Ann M. Dvorak, and James G. Fox

The normal skin and other tissues of adult mast cell–deficient WBB6F$_1$-W/W^v or WCB6F$_1$-Sl/Sl^d mice contain <1.0% the number of mast cells present in the corresponding tissues of the congenic normal (+/+) mice. As a result, genetically mast cell–deficient WBB6F$_1$-W/W^v or WCB6F$_1$-Sl/Sl^d mice are widely used for studies of mast cell differentiation and function. We found that mast cells developed at sites of idiopathic chronic dermatitis in WBB6F$_1$-W/W^v mice and that the number of mast cells present in the skin of WBB6F$_1$-W/W^v mice was proportional to the severity of the dermatitis (in ear skin, there were 33 ± 4 mast cells/mm^2 of dermis at sites of severe dermatitis v 9 ± 3 at sites of mild dermatitis, 0.8 ± 0.3 in skin without dermatitis, and 100 ± 7 in the normal skin of congenic WBB6F$_1$-+/+ mice; in back skin, the corresponding values were 2.0 ± 0.6, 1.1 ± 0.9, 0.025 ± 0.025, and 26.2 ± 3.2). The development of mast cells was a local, not systemic, consequence of the dermatitis. Thus, WBB6F$_1$-W/W^v mice with severe dermatitis lacked mast cells in skin not showing signs of dermatitis and also in the peritoneal cavity, stomach, cecum, and tongue. Idiopathic chronic dermatitis was not associated with the local development of mast cells in WCB6F$_1$-Sl/Sl^d mice, a mutant whose mast cell deficiency is due to a mechanism distinct from that of WBB6F$_1$-W/W^v mice. These findings may have implications for understanding the nature of the mast cell deficiency in WBB6F$_1$-W/W^v and WCB6F$_1$-Sl/Sl^d mice and for the use of these mutants to analyze mast cell differentiation and function.

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KITAMURA AND HIS ASSOCIATES reported that WBB6F$_1$-W/W^v mice, which were known to exhibit macrocytic anemia, sterility, and a lack of hair pigmentation,[1,2] were also profoundly deficient in tissue mast cells.[3] The skin of adult W/W^v mice contains ≤1% the number of mast cells present in the skin of the congenic normal (+/+) mice, and no mast cells whatsoever have been detected in the following organs/sites: bone marrow, spleen, thymus, brain, heart, lung, kidney, bladder, liver, stomach, small intestine, cecum, mesentery, peritoneal cavity, or hindlimb skeletal muscle.[3-8]

The gene products affected by the mutations in W/W^v mice and the precise mechanisms by which these mutations produce mast cell deficiency are unknown.[6,9,10] But it has been demonstrated that W/W^v mice provide a hospitable environment for the differentiation/maturation of mast cells from competent, genetically compatible precursors. These include bone marrow cells derived from WBB6F$_1$-+/+ or C57BL/6-bg^J/bg^J mice as well as cultured, growth factor–dependent mast cells derived from these animals.[3-7,9-11] In addition to demonstrating that the mast cell deficiency of WBB6F$_1$-W/W^v mice reflects a quantitative and/or qualitative abnormality of bone marrow–derived mast cell precursors, the finding that bone marrow cells or cultured mast cells derived from WBB6F$_1$-+/+ mice can restore mast cell populations to W/W^v mice greatly increases the value of these mutants for studies of mast cell biology.[7-11]

In part because a profound deficiency of mast cells has been regarded as a stable aspect of the phenotype of adult WBB6F$_1$-W/W^v mice, these mutants and their normal (+/+) littermates have been used in a wide variety of investigations concerning mast cell function in immediate- and delayed-type hypersensitivity reactions, parasite immunity, acute inflammation, thromboembolic phenomena, reparative processes, and tumor biology.[6,9,10] We now report that W/W^v mice can develop significant populations of cutaneous mast cells in association with a form of idiopathic chronic dermatitis that occurs in mice of the C57BL/6 and related genetic backgrounds. This finding underscores the need to ascertain, in studies using mast cell–deficient mice for evaluation of mast cell biology, whether the particular experimental model used can induce the development of mast cells in the W/W^v mice. It also may have implications for understanding the mechanism of mast cell deficiency in these mutants. Some of our results have been reported in abstract form.[11a]

MATERIALS AND METHODS

Mice. Male mast cell–deficient W/W^v mice and their normal (+/+) littermates (WB/ReJ-W/+ × C57BL/6J-W^v/+)F$_1$-(W/W^v, +/+) and male mast cell–deficient Sl/Sl^d mice and their normal (+/+) littermates (WC/ReJ-Sl/+ × C57BL/6J-Sl^d/+)F$_1$-(Sl/Sl^d, +/+) were purchased from the Jackson Laboratories, Bar Harbor, ME, at 4 to 8 weeks of age. One group of pooled littermate WBB6F$_1$-(W/W^v, +/+) mice and a group of pooled littermate WCB6F$_1$-(Sl/Sl^d, +/+) mice were housed at the Beth Israel Hospital (BIH) Animal Care Facility. Another group of pooled littermate W/W^v and congenic +/+ mice were housed at the Massachusetts Institute of Technology (MIT) Animal Care Facility. Both facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC), and the mice were maintained in accordance with guidelines established by the BIH and MIT Committees on Animal Research and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS publication 86-23, revised 1985).

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Development of chronic dermatitis. Mice of the C57BL/6 and related strains develop a noncontagious and progressive severe dermatitis, apparently unrelated to known infections or nutritional factors.[12,13] Murphy[13] reported that Sl/Sl^d and W^x/W^v mutants were more susceptible to the development of such dermatitis than the congenic normal (+/+) animals. The dermatitis characteristically becomes apparent in older mice and presents as areas of erythema, hair loss, and cutaneous thickening, sometimes associated with the development of ulceration, that involves the skin of the ears and adjacent head, neck, dorsal part of the back, and axilla.[12,13]

Histologic assessment of dermatitis and morphological evaluation and quantitation of mast cells. Mice exhibiting evidence of dermatitis involving the ears and control mice with grossly normal ear skin were killed by cervical dislocation. The ears were then amputated and fixed and processed for 1-μm-thick, Epon-embedded, Giemsa-stained sections[8,14,15] or for transmission electron microscopy[15] as previously described. We also determined whether the mast cells that appeared at sites of idiopathic dermatitis in W/W^v mice stained with berberine sulfate, a fluorescent cationic dye that binds to the heparin in the cytoplasmic granules of mature "connective tissue–type" mast cells such as those normally present in the skin of mice.[7,16,17] For these studies, representative skin specimens were fixed, processed, and stained with berberine sulfate (Sigma Chemical Co, St Louis) as previously described[7] and examined in a Zeiss fluorescence microscope (Carl Zeiss, Inc, Thornwood, NY) equipped for epiillumination. Biopsy samples were also obtained from areas of W/W^v back skin exhibiting evidence of dermatitis as well as from the grossly normal back skin of W/W^v and congenic +/+ mice.

In some cases, additional, grossly normal tissues of W/W^v mice with severe dermatitis were prepared for 1-μm sections (normal back skin, tongue, stomach, cecum), and toluidine blue–stained cytocentrifuge preparations were made of the resident peritoneal cells. Peritoneal cells were collected by lavaging the peritoneal cavity with 3 mL of Hanks' balanced salt solution (HBSS, GIBCO, Grand Island, NY) containing 0.1% bovine serum albumin (Sigma).

For quantitation of mast cell numbers, the coded 1-μm sections were examined by light microscopy at 400×, and the number of mast cells present was recorded.[8] The same slide was then projected onto a piece of paper with a Leitz Type XI C Xenon slide projector (Leitz, Rockleigh, NJ), and the following structures were traced: epidermal surface, dermal-epidermal junction, cartilage (in ear specimens), or panniculus carnosus (in back skin specimens). The area of dermis (mm^2) was determined by analyzing the projected images using a MOP-3 digitizer (Zeiss) interfaced to an HP86 computer (Hewlett-Packard Co, Palo Alto, CA).[18] The area of dermis for ear specimens was taken as the total area of ear minus the sum of the area of epidermis plus the area of cartilage; for back skin specimens, the area of dermis was taken as the total area between the dermal-epidermal junction and the superficial surface of the panniculus carnosus. The number of mast cells was expressed as the number of cells per square millimeter of dermis. Differences in mast cell counts were examined for statistical significance by the Mann Whitney U test (two-tailed).

RESULTS

Mast cells occur at sites of chronic dermatitis in $WBB6F_1$-W/W^v mice. We first examined 1-μm, Epon-embedded, Giemsa-stained sections prepared from W/W^v mouse ears exhibiting gross evidence of dermatitis as well as sections of grossly normal ears from W/W^v and congenic +/+ mice. Based on the histologic findings, the dermatitis was classified as severe or mild (Fig 1). Ears with severe dermatitis exhibited marked epidermal and dermal thickening, dermal infiltration of leukocytes, and numerous mast cells (Fig 1A to E). By transmission electron microscopy (Fig 1E), the ultrastructure of mast cells at sites of severe dermatitis in W/W^v mice closely resembled that reported previously for normal mouse mast cells.[19,20] Moreover, these mast cells stained with berberine sulfate (Fig 1E, insert), a fluorescent dye that binds to the heparin in the cytoplasmic granules of mature skin mast cells.[7,16,17] Ears with mild dermatitis exhibited slight epidermal thickening, little or no dermal thickening, sparse leukocytic infiltration, and occasional mast cells (Fig 1F).

We quantitated dermal mast cells in the ears of W/W^v mice with severe or mild dermatitis as well as in the ears of normal W/W^v or congenic +/+ mice (Table 1). Two different groups of pooled littermate W/W^v mice were analyzed. One group (group A) was housed exclusively at BIH; these mice were killed at ~8 months of age. The other group (B), which included pooled littermate $WBB6F_1$-+/+ mice, was housed exclusively at MIT; these mice were killed at ~15 months of age. Approximately 50% of the W/W^v mice in each group exhibited gross evidence of dermatitis involving at least one ear. By contrast, none of the pooled littermate +/+ mice in group B developed evidence of dermatitis.

In both groups, the ears of representative normal W/W^v mice contained rare dermal mast cells (0.8/mm^2 of dermis). This value, representing <1% of the number of mast cells present in the ears of the congenic +/+ mice, is in good agreement with previous reports.[3,4,7] However, dermal mast cells were increased approximately 11-fold at sites of mild dermatitis and 41-fold at sites of severe dermatitis (both values, $P < .001$ *v* the value in normal ears of W/W^v mice). The number of mast cells in W/W^v mouse ears with severe dermatitis, when expressed as the number per square millimeter of dermis, was significantly less than the number in the ears of +/+ mice (33.0 ± 7.5 *v* 100.0 ± 7.3, $P < .001$). But the distribution of mast cells in W/W^v ears with dermatitis was uneven, with some areas of dermis containing many mast cells and others containing none (Fig 1B to D). Thus, the concentration of mast cells in some regions of W/W^v ears with dermatitis probably approached or exceeded that in the normal ears of the congenic +/+ mice.

The development of mast cells at sites of chronic dermatitis is a local, not a systemic, consequence of the dermatitis. Two findings indicated that the development of mast cells at sites of dermatitis reflects a local consequence of the dermatitis. First, the number of dermal mast cells that developed in the ears of W/W^v mice appeared to depend on the severity of dermatitis. This was true whether all ears exhibiting similar degrees of dermatitis were pooled, as in Table 1, or when the three W/W^v mice that exhibited different degrees of involvement of their two ears were considered separately. The extent of dermatitis (none, mild, severe) and mast cell counts (number per square millimeter of dermis) for the left (L) and right (R) ears of these mice were as follows: mouse 14, L: mild, 24.1, R: none, 1.5; mouse 19, L: severe, 25.2, R: mild, 4.9; mouse 28, L: severe, 33.3, R: mild, 5.2.

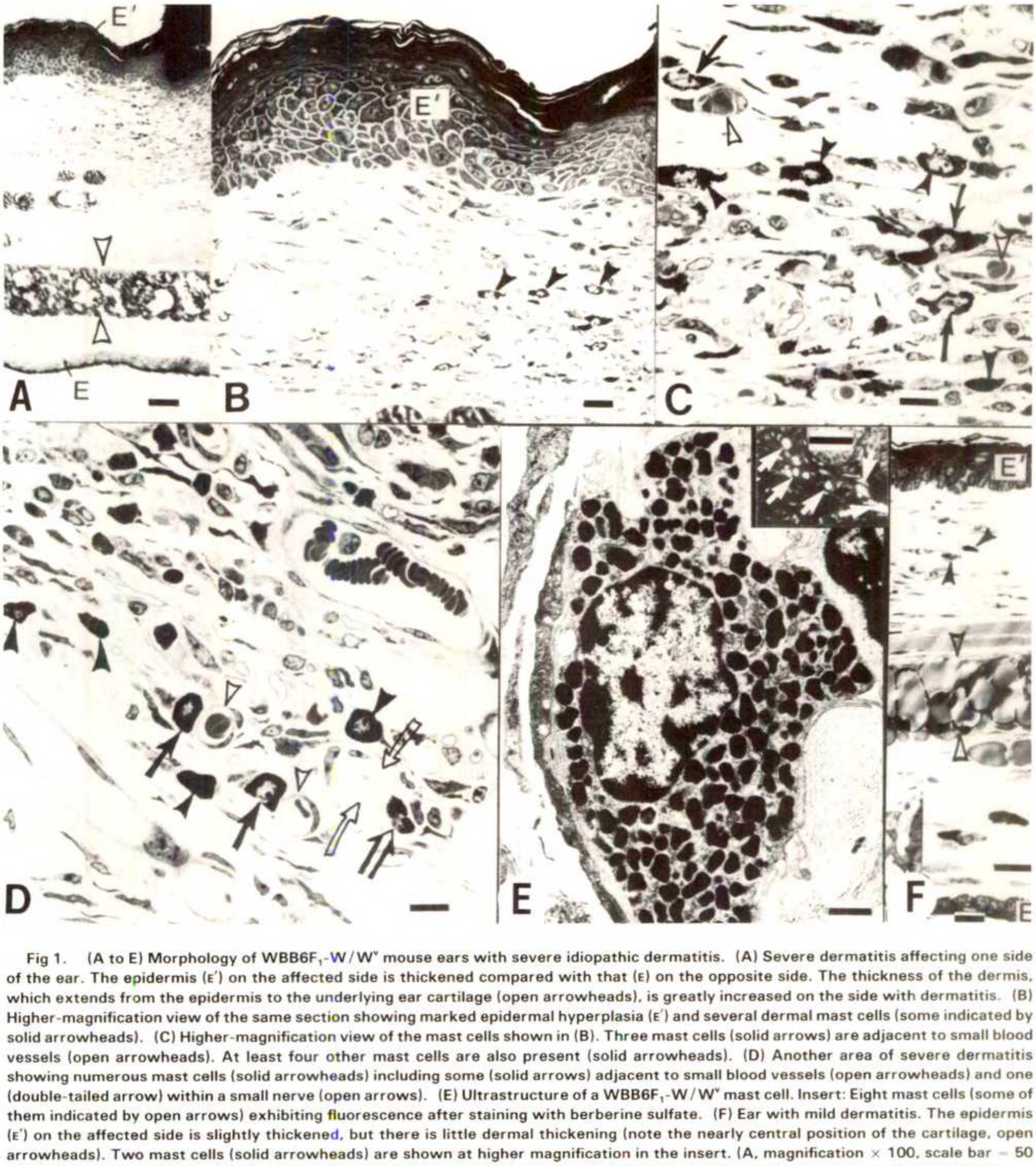

Fig 1. (A to E) Morphology of WBB6F$_1$-W/W^v mouse ears with severe idiopathic dermatitis. (A) Severe dermatitis affecting one side of the ear. The epidermis (E') on the affected side is thickened compared with that (E) on the opposite side. The thickness of the dermis, which extends from the epidermis to the underlying ear cartilage (open arrowheads), is greatly increased on the side with dermatitis. (B) Higher-magnification view of the same section showing marked epidermal hyperplasia (E') and several dermal mast cells (some indicated by solid arrowheads). (C) Higher-magnification view of the mast cells shown in (B). Three mast cells (solid arrows) are adjacent to small blood vessels (open arrowheads). At least four other mast cells are also present (solid arrowheads). (D) Another area of severe dermatitis showing numerous mast cells (solid arrowheads) including some (solid arrows) adjacent to small blood vessels (open arrowheads) and one (double-tailed arrow) within a small nerve (open arrows). (E) Ultrastructure of a WBB6F$_1$-W/W^v mast cell. Insert: Eight mast cells (some of them indicated by open arrows) exhibiting fluorescence after staining with berberine sulfate. (F) Ear with mild dermatitis. The epidermis (E') on the affected side is slightly thickened, but there is little dermal thickening (note the nearly central position of the cartilage, open arrowheads). Two mast cells (solid arrowheads) are shown at higher magnification in the insert. (A, magnification × 100, scale bar = 50 μm; B and F, magnification × 250, scale bars = 20 μm; C, D, and insert in F, magnification × 630, scale bars = 10 μm; E, magnification × 7,000, scale bar = 1 μm; insert in E, magnification × 140, scale bar = 50 μm.) A to D and F, 1-μm-thick, Epon-embedded, Giemsa-stained sections.

We also selected four W/W^v mice that had dermatitis and high mast cell counts in at least one ear (range, 20.3 to 57.7 mast cells/mm^2 of dermis) and examined multiple other anatomic sites or organs for the presence of mast cells. No mast cells whatsoever were detected in the normal back skin, tongue, forestomach, glandular stomach, cecum, or peritoneal cavity of these mice.

The number of mast cells at sites of chronic dermatitis varies according to anatomic site. Many of the W/W^v mice analyzed in Table 1 exhibited dermatitis involving the cephalad regions of the back as well as the ears. We therefore sampled the affected and unaffected back skin of these mice for histologic analysis and mast cell counts. Dermal mast cell counts in the normal back skin of both W/W^v and congenic

Table 1. Dermal Mast Cell Counts (per Square Millimeter of Dermis) in the Ears of Normal WBB6F$_1$-W/W^v and WBB6F$_1$-+/+ Mice and in WBB6F$_1$-W/W^v Mice With Idiopathic Dermatitis

Genotype	Dermatitis*	Group A (8 mo old)	Group B (15 mo old)	Combined A + B
W/W^v	None	1.0 ± 1.0 (3)	0.7 ± 0.4 (7)	0.8 ± 0.3 (10)
W/W^v	Mild	3.2 ± 1.4 (4)	10.2 ± 3.3 (16)	8.8 ± 2.7 (20)
W/W^v	Severe	32.7 ± 5.1 (7)	33.2 ± 4.7 (14)	33.0 ± 3.5 (21)
+/+	None	ND	100.0 ± 7.3 (16)	100.0 ± 7.3 (16)

Mast cell counts were performed on 1-μm, Epon-embedded, Giemsa-stained sections of ears, and the area of dermis was calculated by planimetry of the projected image of the slide (see the text for details). The data (no. of mast cells per square millimeter of dermis) are presented as mean ± SEM (no. of ears). In group A, the mast cell counts in ears with mild dermatitis were not significantly different ($P < 0.05$) than those in normal ears of W/W^v mice. All other comparisons within group A, group B, or the combined group were significant at the $P < .001$ level by the Mann Whitney U test (two-tailed).

Abbreviation: ND, not determined.

*The extent of dermatitis was assessed histologically.

+/+ mice (Table 2) were lower than those in the ear skin of mice of the same genotype (Table 1). At sites of severe dermatitis, the numbers of dermal mast cells in W/W^v mice were significantly elevated compared with the baseline levels (2.0 ± 0.6 *v* 0.025 ± 0.025 mast cells/mm^2 of dermis, an 80-fold increase). Nevertheless, the number of mast cells at sites of severe dermatitis involving the back skin was only 6% of the corresponding figure for ear skin with severe dermatitis (2.0 *v* 33.0 mast cells/mm^2, respectively, $P < .001$).

Chronic dermatitis is not associated with the local development of mast cells in WCB6F$_1$-Sl/Sl^d mice. Like WBB6F$_1$-W/W^v mice, WCB6F$_1$-Sl/Sl^d mice are semisyngeneic to C57BL/6 mice,[3,6,21] and Sl/Sl^d and W/W^v mice are similar in phenotype: both have a macrocytic anemia, lack cutaneous melanocytes, are sterile, and exhibit a profound deficiency in mast cells.[1,2,21] Sl/Sl^d mice also spontaneously develop chronic dermatitis.[13] However, the mechanisms of mast cell deficiency in W/W^v and Sl/Sl^d mice are different.[3,6,21] W/W^v mice have a defect of hematopoietic stem cells (CFU-S)[22,23]; their mast cell deficiency (and anemia) can be repaired by intravenous (IV) transplantation of bone marrow cells derived from the congenic +/+ mice.[3,6] By contrast, Sl/Sl^d mice have apparently normal hematopoietic stem cells.[24] The mast cell deficiency of this mutant is thought to reflect an abnormality in the microenvironmental factors promoting mast cell differentiation/maturation,[6,21] and neither the anemia nor the mast cell deficiency of Sl/Sl^d mice can be cured by IV transplantation of the congenic +/+ bone marrow cells.[21]

Twelve Sl/Sl^d mice were sacrificed at 12 months of age: 2 had no evidence of dermatitis, 3 had mild dermatitis, and 7 had severe dermatitis involving at least one ear and/or the adjacent neck and back skin. Virtually no mast cells were identified in the ear or back skin of Sl/Sl^d mice with dermatitis (Table 3). In other respects (extent of epidermal and dermal changes, inflammatory cell infiltration), the histologic features of the dermatitis in Sl/Sl^d and W/W^v mice were similar.

Table 2. Dermal Mast Cell Counts in Back Skin of Normal WBB6F$_1$-W/W^v and WBB6F$_1$-+/+ Mice and in WBB6F$_1$-W/W^v Mice With Idiopathic Dermatitis

Genotype	Dermatitis	No. Mast Cells/mm^2 of Dermis
1. W/W^v	None	0.025 ± 0.025 (16)
2. W/W^v	Mild	1.1 ± 0.9 (5)
3. W/W^v	Severe	2.0 ± 0.6 (18)*
4. +/+	None	26.2 ± 3.2 (4)†

Mast cell counts were performed as described in the legend to Table 1 (dermis equals the area from the dermal-epidermal junction to the superficial surface of the panniculus carnosus). The data, presented as mean ± SEM (no. of biopsy samples), are from the same mice shown in Table 1 (combined groups A and B).

*$P < .001$ *v* values for lines 1 or 4.

†$P < .001$ *v* values for lines 1, 2, or 3.

DISCUSSION

We found that large numbers of mast cells developed at sites of idiopathic chronic dermatitis in W/W^v mice. Several lines of evidence indicated that the development of mast cells at a site of dermatitis was a local, not a systemic, effect of the dermatitis. In ears with dermatitis, the number of dermal mast cells was greater in ears with severe dermatitis than in ears with mild dermatitis, and the greatest concentrations of mast cells in individual ears were at the sites exhibiting the most severe dermatitis. In a mouse with unilateral dermatitis, only the affected ear exhibited increased numbers of mast cells. Finally, no mast cells were detected in the normal skin, forestomach, glandular stomach, cecum, or peritoneal cavity of four W/W^v mice that had large numbers of mast cells at a site of severe dermatitis.

Although chronic dermatitis was associated with significantly elevated dermal mast cell counts in both the affected ears and back skin of W/W^v mice, the elevation of mast cell numbers was greater in the ears than in back skin. Thus, mast cell counts were 33.0/mm^2 of dermis in W/W^v mouse

Table 3. Dermal Mast Cell Counts in Normal WCB6F$_1$-Sl/Sl^d and WCB6F$_1$-+/+ Mice and in WCB6F$_1$-Sl/Sl^d Mice With Idiopathic Dermatitis

		No. Mast Cells/mm^2 of Dermis	
Genotype	Dermatitis	A. Ear	B. Back Skin
1. Sl/Sl^d	None	0 ± 0 (4)	0.03 ± 0.03 (13)*
2. Sl/Sl^d	Mild	0 ± 0 (8)	0 ± 0 (3)
3. Sl/Sl^d	Severe	0.03 ± 0.03 (10)*	0 ± 0 (2)
4. +/+	None	103.5 ± 9.8 (10)†	33.3 ± 2.6 (8)†

Mast cell counts were performed as described in the legends to Tables 1 and 2. The data are presented as mean ± SE (no. of ears or back skin biopsy samples).

*These values reflect the identification of a single mast cell in each of the two groups of specimens.

†$P < .001$ *v* any of the other values in columns A or B including the other value for +/+ mice.

ears with severe dermatitis (33.0% of the corresponding value for normal +/+ mice) v 2.0/mm^2 of dermis in W/W^v mouse back skin with severe dermatitis (7.6% of the corresponding value for normal +/+ mice). In part, this finding may be related to the fact that back skin normally contains fewer mast cells than ear skin (26.2 v 100 mast cells/mm^2 of dermis in normal +/+ mice [$P < .001$], 0.025 v 0.8 mast cell/mm^2 of dermis in normal W/W^v mice [$P < .1$]). If the differences in the baseline mast cell counts in the normal ear and back skin of W/W^v mice are taken into account, the elevations of mast cell numbers associated with chronic dermatitis are similar in the two anatomic sites (a 41-fold elevation over the normal value in the ear and an 80-fold elevation over the normal value in the back skin). The factors regulating the normal numbers of dermal mast cells at different anatomic sites are not clear, but our data suggest that even the apparently normal ears of old W/W^v mice are not as profoundly mast cell deficient as is the back skin of the same mice. There may be an interesting parallel to this finding in the effect of certain W locus mutations on melanocytes. Most mice with a double dose of mutations at the W locus totally lack cutaneous melanocytes.[25] But certain of these mutants (eg, W^f/W^v, W^v/W^v) routinely or occasionally exhibit some pigmentation of the ear skin.[25]

The etiology of the chronic dermatitis that occurs in mice of the C57BL/6 and related strains is unknown.[12,13] Attempts to identify conventional infectious agents have been unsuccessful,[12,13] and neither histologic nor microbiologic examination (data not shown) of the mice used in our experiments revealed any clues concerning the pathogenesis of the problem. In accord with previous studies of Sl/Sl^d and W^v/W^v mice, we found that our W/W^v or Sl/Sl^d mice developed a higher incidence of idiopathic dermatitis than did the congenic +/+ mice. Whether the increased susceptibility of the mutants for the development of idiopathic dermatitis has any relationship to the mast cell deficiency expressed by W/W^v and Sl/Sl^d mice is unclear.

The findings reported here are important for two reasons. First, they offer a new perspective concerning the nature of the mast cell abnormality expressed by W/W^v mice. Although it is well known that the tissues of W/W^v mice permit mast cell differentiation/maturation from competent precursor cells such as those derived from congenic +/+ mice,[3-7] our data represent clear evidence that the mast cell deficiency of $WBB6F_1$-W/W^v mice can undergo local self-repair in vivo. This finding must reflect a response of the small number of mast cells present in the normal skin of W/W^v mice and/or W/W^v mast cell precursors to a dermatitis-associated alteration of the W/W^v skin microenvironment. It is of interest, in this context, that several investigators have reported that mast cells of W/W^v origin can be generated in vitro when hematopoietic cells of W/W^v mice are cultured in media containing high levels of interleukin 3 and other growth factors.[7,26,27] Moreover, such in vitro analysis indicates that the relative concentrations of mast cell precursors in the bone marrow, spleen, and peripheral blood of W/W^v mice are similar to those in the congenic +/+ mice.[27] These findings raise the possibility that one microenvironmental alteration at the sites of chronic dermatitis that might contribute to the local development of W/W^v mast cells is the production of growth factors similar to those present in the in vitro systems.

On the other hand, W/W^v mast cells generated in suspension cultures exhibited diminished proliferative potential in vitro[26] and failed to survive and mature after injection into normal W/W^v mice in vivo.[7] By contrast, many of the mast cells present at sites of idiopathic dermatitis in W/W^v mice were mature according to light and electron microscopic criteria and as judged by staining of the cytoplasmic granules with berberine sulfate. Thus, the local alterations produced in association with chronic idiopathic dermatitis resulted in both the marked elevation of the number of dermal mast cells and the expression, by these cells, of a mature phenotype. These effects might reflect the continuous local production of soluble mast cell growth/maturation factors and/or other significant alterations at the site of dermatitis. But whatever the reason(s) for mast cell development at sites of idiopathic dermatitis, elucidation of the mechanisms responsible for this effect is likely to provide additional insight into the nature of the mast cell defect expressed by W/W^v mice.

The second important implication of our findings concerns the use of genetically mast cell–deficient mice to study mast cell function in vivo. W/W^v mice are widely used to analyze the roles of mast cells in diverse biologic responses.[6,9,10,28] Our findings show that it is critical to search for the "spontaneous" appearance of mast cells at sites of inflammatory or pathological processes under investigation in W/W^v mice, particularly in those studies involving models of chronic disease processes. It is of interest that the changes associated with the idiopathic dermatitis that occurred in Sl/Sl^d mice apparently were unable to alter significantly the microenvironmental defect thought to be responsible for the failure of mast cell differentiation/maturation in this mutant.[21] This result indicates that for certain studies of mast cell biology $WCB6F_1$ (-Sl/Sl^d, -+/+) mice may represent a more appropriate choice than the $WBB6F_1$ (-W/W^v, -+/+) animals.

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The Proliferative Response of B Cell Chronic Lymphocytic Leukemia to Interleukin 2: Functional Characterization of the Interleukin 2 Membrane Receptors

By Ivo Touw, Lambert Dorssers, and Bob Löwenberg

To determine the growth properties of B cell chronic lymphocytic leukemia (B CLL) and to identify possible abnormalities thereof, we examined the in vitro action of interleukin 2 (IL2) in four patients. Using radiolabeled IL2 and monoclonal antibodies reactive with IL2 membrane receptors we show that CLL cells, after their activation in vitro, express IL2 receptors of a high- as well as a low-affinity type, exactly as has been reported for normal T and B blasts. In three of the four reported cases, CLL proliferation (measured with ^{3}H-thymidine incorporation) depended on the addition of phytohemagglutinin (PHA) to activate the cells and IL2 (optimal concentration, 10 to 100 U IL2/mL). In contrast, the cells of the fourth case of CLL (CLL-4) proliferated in an autonomous fashion, ie, without a need for PHA and IL2 in culture. Specific blocking of the IL2-binding sites with anti-IL2 receptor monoclonal antibodies almost completely inhibited the proliferation of these cells, which indicated that functional IL2 receptors were required for the autonomous proliferation. The demonstration of low concentrations of IL2 activity in the culture medium conditioned by the cells suggests that endogenous IL2 had been responsible for the spontaneous ^{3}H-thymidine uptake by the CLL cells of patient 4. However, we were unable to extract IL2 mRNA from the cells (neither fresh nor after various in vitro incubations) in quantities detectable by Northern blot analysis that would prove that the CLL cells of patient 4 were actively synthesizing IL2 during culture. Thus, individual cases of B CLL are subject to variable growth regulation involving functional IL2 receptors on the cell surface: (1) after activation with PHA the cells respond to exogenous IL2 in a fashion similar to normal B lymphocytes, or (2) the cells are stimulated by endogenous IL2 (or an IL2-like activity) and do not require activation with PHA.

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INTERLEUKIN 2 (IL2) is one of the polypeptide hormones controlling the proliferation and differentiation of B cells via surface membrane receptors.[1-10] Studies in our laboratory have focused on the role of IL2 in the proliferation of leukemic B cells, one of the purposes being to analyze whether the leukemic cells express abnormal responsiveness to the hormone.[11] Using a colony culture technique, we have shown that in acute lymphoblastic leukemia (ALL) of B cell origin, ie, common ALL and pre-B ALL, IL2 stimulates proliferation, although only when additional factors derived from leukocyte feeder cells are present in culture as well.[12,13] The nature of the latter factors has not been established yet. In B cell chronic lymphocytic leukemia (B CLL), IL2 as a single stimulus induces in vitro colony formation provided that the CLL colony-forming cells are activated with the phorbol ester 12-0-tetradecanoyl phorbol-13-acetate (TPA) or the lectin phytohemagglutinin (PHA).[14] Proliferation of CLL cells stimulated by IL2 and TPA has also been reported in ^{3}H-thymidine (^{3}H-TdR) incorporation assays.[15]

In the present study we characterize the function of the IL2 membrane receptors of B CLL cells by using a radiolabeled IL2 preparation and further analyze IL2 responsiveness in microculture. We demonstrate that in vitro activated CLL cells express two classes of IL2 receptors, one with a high affinity and one with a low affinity for IL2, a property that these cells share with normal activated T and B blasts.[5] Results from the microculture experiments show that CLL cells of three patients require IL2 and activation by PHA for proliferation. On the other hand, in a fourth case, CLL cells proliferate without supplementation of IL2 and PHA to the cultures. The results of additional experiments suggest that the "spontaneous" proliferation of these CLL cells is in fact not IL2 independent but controlled by endogenous IL2.

MATERIALS AND METHODS

Leukemic cells. CLL cells were isolated by Ficoll-Isopaque (Nyegaard, AS, Oslo) separation from the peripheral blood of patients who had not received cytoreductive therapy for at least 12 weeks.[16] All patients had given informed consent. T lymphocytes were removed by rosetting with 2-aminoethylthiouronium bromide (AET)-treated sheep erythrocytes followed by a second Ficoll-Isopaque separation.[17]

The T cell–depleted leukemic cell fractions contained 97% to 100% small lymphocytes and always less than 0.5% E rosette–forming cells. Peripheral blood leukocyte counts and immunotypes are listed in Table 1.

Cell cultures. Proliferation of CLL cells was measured by ^{3}H-TdR uptake. Cells (1×10^5) were cultured in triplicate in round-bottom microtiter wells (Greiner, Alphen a/d Rijn, The Netherlands) in 100 μL culture medium. This culture medium consisted of Dulbecco's modified Eagle's minimal essential medium, heat-inactivated fetal calf serum (6.7% vol/vol), heat-inactivated horse serum (6.7% vol/vol), trypticase soy broth (6.7% vol/vol) supplemented with dialyzed bovine serum albumin (0.75% wt/vol), egg lecithin (3×10^{-5} mol/L), Na_2SeO_3 (10^{-7} mol/L), iron-saturated human transferrin (7.7×10^{-6} mol/L), and β-mercaptoethanol (10^{-4} mol/L) as modified from Guilbert and Iscove.[18] Additions to the cultures included 0.1% vol/vol PHA (reagent grade, Wellcome Reagents, Dartford, England), pure recombinant IL2 with a specific activity of 1.9×10^6 U/mg protein or approximately

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Table 1. Hematologic Data of CLL Patients

Patient	Peripheral Blood Leukocyte Count (× 10 ^{9}/L)	Immunotype	
		Ig Heavy Chain	Ig Light Chain
CLL-1	379	M	λ
CLL-2	161	M + D	κ
CLL-3	268	M + D, A, G	κ
CLL-4	152	M + D	λ

3 × 10^{13} U/mol (Biogen SA, Geneva), and monoclonal antibodies (MoAb) reactive with IL2 membrane receptors (see the like-named section). Cultures were performed in triplicate. Sixteen hours before harvesting the cells (Titertek cell harvester 550; Flow Laboratories, Irvine, Scotland), 0.1 μCi of ^{3}H-TdR (Amersham Corp, UK; specific activity, 2 Ci/mmol) was added to each microwell. Radioactivity was measured by liquid scintillation counting (Beckman LS 3800, Beckman Instruments, Fullerton, CA).

Suspension cultures of CLL cells, normal peripheral blood mononuclear cells, and Jurkat cells were performed in test tubes (1) to assess IL2 activities elaborated in the culture medium (2) to extract RNA from the in vitro stimulated cells, and (3) to determine binding of radiolabeled IL2 to in vitro stimulated cells. Cells (2 × 10^6/mL culture medium) were incubated either with PHA (0.1% vol/vol) plus TPA (10 ng/mL), PHA alone, or without additions. After various incubation periods (ranging from four hours to seven days) followed by centrifugation, the culture supernatants were collected, filter sterilized (0.22-μm filter, Millipore, Bedford, MA) and stored at −20°C. For the purpose of RNA extraction, the cells were washed twice with Hanks' balanced salt solution (HBSS), collected in Eppendorf microcentrifugation tubes, quickly frozen (30 seconds) in liquid nitrogen and stored at −80°C. For radiolabeled IL2-binding experiments, the cells were incubated for one hour in fresh culture medium and washed five times with ice-cold α-medium supplemented with 1% wt/vol bovine serum albumin (BSA).

IL2-producing Jurkat cells (a gift from Dr L.A. Aarden, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam) were cultured in RPMI medium supplemented with 10% heat-inactivated FCS in T75 culture flasks (Greiner). For induction of IL2 mRNA the cells were cultured in the presence of PHA (1% vol/vol) and TPA (10 ng/mL) for six hours. After two washings with HBSS, the cells were collected for RNA extraction, frozen in liquid nitrogen, and stored at −80°C as described earlier.

Murine IL2-dependent (CTLL) cells (a gift from S. Knaan-Shanzer, Radiobiological Institute TNO, Rijswijk, The Netherlands) were cultured as described.[19] To estimate IL2 concentrations in CLL culture supernatants, 10%, 20%, and 40% vol/vol of these supernatants were tested for their stimulatory effects on the proliferation of the CTLL cells. These estimations were compared with the proliferative response of the CTLL cells to titrated recombinant IL2 concentrations.

Cytogenetic analysis. Spread metaphases of cultured CLL cells were checked for cytogenetic abnormalities by using Q-, R-, and G-banding techniques.[20] These analyses were performed by Dr A. Hagemeijer and E.M.E. Smit (Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands).

MoAbs reactive with IL2 receptors. Four MoAbs reactive with membrane receptors for IL2 were used: three of these, ie, mouse MoAbs anti-IL2 receptor (Becton Dickinson, Mountain View, CA) and anti-TAC[21] and rat MoAb 18 E 64[22] compete with IL2 for binding to the receptor. The fourth, rat MoAb 36 A 1.2, has been shown not to interfere with IL2 binding.[22]

Indirect immunofluorescence. For immunologic detection of IL2 membrane receptors, cells were treated with anti-IL2 receptor MoAb and with goat antimouse immunoglobulin coupled with fluorescein isothiocyanate (GAM/FITC, Nordic Immunology, Tilburg, The Netherlands) and analyzed on a fluorescence activated cell sorter (FACS 440, Becton Dickinson, Sunnyvale, CA) as described.[12]

RNA extractions, Northern blotting, and hybridization with cDNA probes. RNA was extracted by two different methods yielding comparable amounts and quality of total RNA, ie, hot phenol extraction (in the presence of vanadyl-ribonucleoside complexes to inhibit RNase activity) and the guanidinium/cesium chloride method.[23] Poly(A)+ RNA was isolated by paper affinity chromatography using poly(U)-coated paper (Medac, Hamburg, Germany).[24] RNA was electrophoresed on formaldehyde-agarose gels[21] and blotted onto nylon hybridization membrane (Gene Screen Plus, New England Nuclear, Boston). The human IL2 cDNA used for hybridization was obtained from TPA-stimulated Jurkat cells and cloned in a G-tailed pBR322 vector (prepared and kindly supplied by Dr H. Pannekoek, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam). The gel-purified IL2 cDNA fragment containing nucleotides 377 to 837[25] was isolated from the vector by digestion with *Pst*I (Boehringer, Mannheim, Germany) followed by polyacrylamide gel electrophoresis of the digest. For control hybridizations, the RNA blots were rehybridized with hamster actin cDNA *Pst*I-*Xba*I fragment containing the coding region.[26] The IL2 and actin cDNA fragments were radioactively (^{32}P) labeled by random priming using oligodeoxynucleotides, essentially according to Feinberg and Vogelstein.[27] Hybridizations were carried out as described.[23] Autoradiography using Kodak Xomat AR x-ray film occurred at −70°C in the presence of an intensifying screen.

Binding of radiolabeled IL2. Before treatment with radiolabeled IL2, the cells were incubated (one hour at 37°C) in fresh culture medium and washed (five times with α-medium containing 1% wt/vol BSA) to remove endogeneous IL2.[24] Then, 10^6 cells in 50 μL α-BSA medium were placed in wells of medium-prewetted (α-BSA), millititer 96-well, 5.0-μm filter-bottom plates (Millipore). To determine the amount of specific binding, serial dilutions of pure, recombinant ^{125}I-IL2 (Amersham, UK) ranging from 6.25 to 800 pmol/L were added to the cells. The cells were incubated with the radiolabeled IL2 for one hour at 37°C. These conditions allow for optimal saturation of high- and low-affinity binding sites.[28] To correct for nonspecific binding of the ^{125}I-IL2, incubations were also performed in the presence of excess (ie, thousandfold concentrations) of nonlabeled recombinant IL2. All incubations were carried out in duplicate. At the end of the incubation, the medium containing the unbound radiolabeled IL2 was sucked away by placing the filtration plates on a vacuum filtration holder (Millipore Filter System). Duplicate samples from the incubation medium were taken to determine unbound radioactivity. The cells retained by the filter bottoms were then washed twice with 100 μL ice-cold α-BSA medium. These washings were carried out rapidly (two to five seconds per wash) to prevent dissociation of specific IL2 binding. After drying (30 minutes at room temperature) the filter bottoms were punched out and collected in gamma counter vials. Radioactivity was counted in a 4/200 automatic gamma counter (Micromedic System, Horsham, PA). The data were analyzed by using the Ligand computer program of Munson and Rodbard.[29]

RESULTS

Expression of IL2 receptors on the CLL cells and binding of radiolabeled IL2. IL2 receptors, as assessed in indirect fluorescence with MoAb aIL2r and flow cytometry, were absent or weakly expressed on the fresh CLL cells. In all cases IL2 receptors were maximally expressed on the cells after 18 hours of suspension culture in the presence of 0.1%

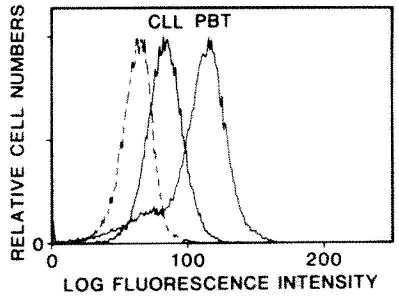

Fig 1. Flow cytometric fluorescence (histogram) analysis of IL2 receptors on activated CLL cells (CLL – 3) and activated normal peripheral blood T lymphocytes (PBT). After 36 hours of in vitro incubation in the presence of 0.1% vol/vol PHA and washings, cells were stained with MoAb aIL2r and GAM/FITC. The dotted curve represents the fluorescence profile of the cells stained with GAM/FITC alone. Staining with a nonrelevant MoAb of the same immunoglobulin subclass as MoAb aIL2r (IgG1) revealed a fluorescence profile identical to that of the GAM/FITC control.

PHA. Flow cytometric histogram analysis indicated that IL2 receptor densities on the in vitro activated CLL cells were lower than on PHA-activated normal T lymphocytes (Fig 1). A binding assay using ^{125}I-IL2 was performed to determine the affinity and numbers of IL2 receptors on the activated CLL cells. For comparison, IL2 receptors on activated T lymphocytes were also studied. Specific binding of the radiolabeled IL2 was measured at concentrations ranging from 6.25 to 800 pmol/L. Scatchard plot analysis (Fig 2) indicated that CLL cells expressed two classes of receptors similar to the T cells, ie, one with an affinity for IL2 in the picomolar range and one with an affinity in the nanomolar range. The data of these experiments are listed in Table 2.

Proliferative response of CLL cells to IL2. DNA synthesis by CLL cells in culture was estimated by ^{3}H-TdR incorporation at various time points for 11 days. Cells were cultured under different stimulatory conditions, ie, in the presence of PHA (0.1% vol/vol) and IL2 (50 U/mL), PHA alone, IL2 alone, and without these additions (Fig 3). In CLL cases 1, 2, and 3, PHA and IL2 were required for induction of proliferation. Omission of one or both of these components resulted in significantly less or no incorporation of ^{3}H-TdR. ^{3}H-TdR uptake was significantly reduced, ie, by 45% to 77% when anti-IL2 receptor MoAb interfering with the IL2 binding had been added to the cultures (at their initiation). This confirmed that stimulation occurred through IL2–IL2 receptor interaction.

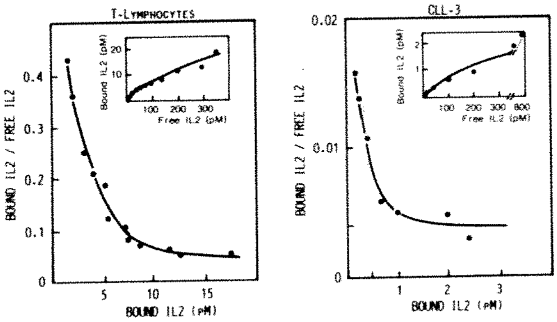

Fig 2. Specific binding of ^{125}I-IL2 to activated normal peripheral T lymphocytes and activated CLL cells (patient 3). T cells and CLL cells had been incubated in the presence of 0.1% vol/vol PHA for 36 hours. The data are expressed as Scatchard plots; the insets show the relationships between the concentrations of free and specifically bound ^{125}I-IL2.

Table 2. Binding of Radiolabeled IL2

Cell Type*	High-Affinity Binding† Kd (pmol/L)	High-Affinity Binding† Mean Number of Receptors per Cell	Low-Affinity Binding† Kd (nmol/L)	Low-Affinity Binding† Mean Number of Receptors per Cell
CLL-1	34	90	ND	ND
CLL-2	7.5	64	ND	ND
CLL-3	28	13	25	13.0 × 10^3
CLL-4	1.2	11	62.5	20.5 × 10^3
Normal T lymphocytes‡				
Donor 1	10.4	171	28.6	41.0 × 10^3
Donor 2	19	154	ND	ND

Abbreviation: ND, not determined.

*Cells had been cultured for 72 hours in the presence of 0.1% vol/vol PHA.

†Estimated using the Ligand computer program of Munson and Rodbard.[29]

‡Isolated from normal peripheral blood.

In contrast, ^{3}H-TdR incorporation by the cells of CLL-4 was completely independent of the addition of PHA and IL2 to the microcultures.

To ensure that the proliferating cells of the four patients were indeed the leukemic cells (and not, eg, residual normal T lymphocytes) immunologic analysis was performed. From this it appeared that on day 7 of culture the cells expressed the clonal phenotypes characteristic of the neoplasm (Table 1). The cultured cells contained less than 1% T cells, as assessed by E rosetting and MoAb anti-T3 labeling in indirect immunofluorescence. Thus, T cells did not significantly contaminate the in vitro growth of the CLL cells. Leukemic cell growth was also confirmed by cytogenetic analysis. On day 7 of culture of the cells of CLL-1, 13 of 15 of the spread metaphases expressed abnormal karyotypes

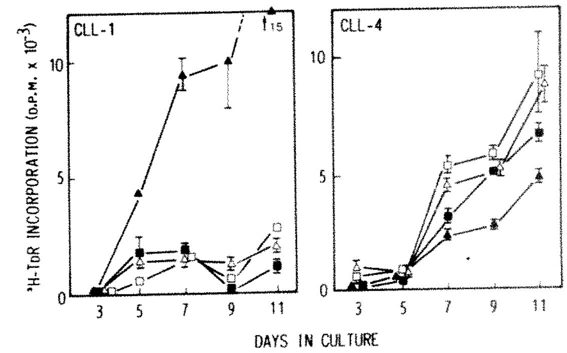

Fig 3. ^{3}H-TdR incorporation by CLL cells under different stimulatory conditions in microculture. (▲ - ▲), PHA (0.1% vol/vol) + 50 μL IL2 (50 U/mL); (■ - ■), PHA alone; (△ - △) rIL2 alone; and (□ - □), no additions. Left panel shows the data of the experiments with CLL – 1 Comparable results were obtained with the cells of CLL – 2 and CLL – 3. In the right panel, the data of ^{3}H-TdR uptake of CLL – 4 are plotted. The cells were cultured for 11 days.

including inv.(X), 22p+, and +22. In CLL-4, 30 of 31 analyzed metaphases appeared abnormal (ie, expressing 14q+) after seven days of culture.

IL2 dependence was investigated by adding IL2 in increasing concentrations to the microcultures (Fig 4). In CLL-1 to -3, optimal proliferation occurred in the presence of 50 to 100 U IL2/mL. On the other hand, in CLL-4, maximal incorporation of ^{3}H-TdR was obtained in cultures without any addition of IL2. This could suggest that the cells of CLL-4 lacked IL2 dependence or that the cells produced IL2 and that proliferation was stimulated by endogenous IL2 (see the next paragraph). On several occasions we attempted to establish CLL cell lines by prolonged culturing and refeeding of the cultures but had negative results.

Analysis of the "spontaneous" proliferation of CLL cells (case 4). In CLL-1 to -3, proliferation depended on the presence of PHA and IL2. In contrast, in CLL-4 the rate of proliferation reached a maximum although no IL2 (and PHA) had been supplemented to the cultures (Figs 3 and 4). Epstein-Barr virus nuclear antigen was absent in these cells, which indicated that these cells had not been transformed by Epstein-Barr virus. We addressed the question as to whether spontaneous proliferation in CLL was truly IL2 independent or possibly mediated through endogenous IL2.

First, we examined the effect of four different MoAbs, reactive with IL2 receptor determinants, on the proliferation of the cells of CLL-4 (Table 3). The addition of MoAbs aIL2r, anti-Tac, and 18 E6.4, which compete with IL2 for receptor binding, completely inhibited ^{3}H-TdR incorporation. In addition, incubation of the cells with MoAb 36 A1.2, an anti-IL2 receptor antibody that does not block IL2 binding, did not exert an inhibitory effect on proliferation. These results indicated that the IL2 receptors (and more specifically the IL2-binding sites of these receptors) had a role in the proliferation even though the cells had not been exposed to exogenous IL2. We hypothesized that these receptors were activated by endogenously produced IL2. To test this possibility, IL2 activities elaborated by the CLL-4 cells in the culture medium were estimated. Media conditioned by CLL cells in suspension cultures were collected at various times (one to seven days) and assayed on a murine IL2-dependent (CTLL) cell line. IL2 activities (ie, at concentrations varying from 0.15 to 1.5 U/mL) were indeed detectable in these CLL cell–conditioned media. By comparison, PHA- and TPA-activated Jurkat cells produced 50 to 100 U IL2/mL culture medium.

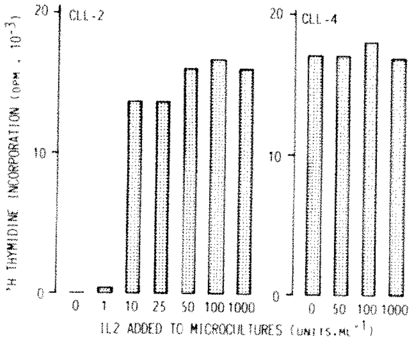

Fig 4. **^{3}H-TdR incorporation by CLL cells in relation to the concentration of IL2 added to the cultures. The cells were incubated with or without IL2 for ten days. Left section, CLL – 2 (cells were cultured in the presence of 0.1% vol/vol PHA); right section, CLL – 4 (cells were cultured in the absence of PHA).**

Table 3. Effect of Four Different Anti-IL2 Receptor MoAbs on the Spontaneous Proliferation of CLL Cells (CLL-4)

MoAb*	Blocking of IL2 Binding	^{3}H-TdR Incorporation (dpm × 10^{-3})†
—		4.30 ± 0.29 (100)
aIL 2 r	+	0.04 ± 0.01 (0.8)
Anti-Tac	+	0.02 ± 0.01 (0.4)
18 E 6.4	+	0.12 ± 0.02 (2.9)
36 A 1.2	—	4.31 ± 0.67 (100.3)

IL2 (or PHA) was not added to the cultures.

*Each of the MoAbs was added at saturating concentrations (as assessed in indirect immunofluorescence) to microcultures at the initiation of the cultures.

†Assessed on day 8 of culture. Data are expressed as means ± SD of triplicate cultures. Figures in parentheses represent the percentages of control values.

Finally, we applied Northern analysis by using an IL2 cDNA probe to test whether IL2 mRNA was present in the cells of CLL-4 (fresh and after various in vitro incubations), which would indicate that the cells were capable of synthesizing IL2. We were repeatedly unable to detect IL2 mRNA in the cells of CLL-4, even when relatively large quantities (ie, estimated at 2 to 5 μg) of purified, poly(A)+, mRNA preparations had been loaded onto the gels (Fig 5). At the same time, IL2 mRNAs from control cells, ie, PHA/TPA-activated Jurkat cells, as well as from PHA-TPA-activated normal peripheral blood T cells, were readily demonstrated (Fig 5). Control hybridization of the blot with actin cDNA indicated that the RNA extracted from the CLL cells had been intact. The inability to show IL2 mRNA in cell samples of CLL-4 could suggest that the endogenous IL2 in the

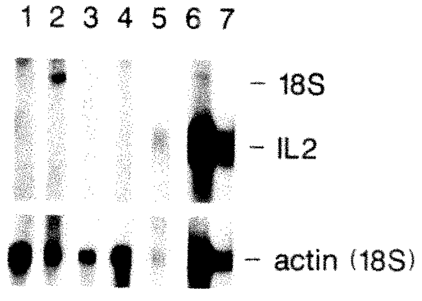

Fig 5. **Autoradiographs of blotted poly(A) + RNA samples from CLL cells, Jurkat cells, and peripheral blood T lymphocytes. The blot was first hybridized with a radioactive IL2 cDNA probe (upper section). After removal of the IL2 cDNA by highly stringent washings, the blot was rehybridized with actin cDNA (lower section). Ribosomal RNA (28S and 18S) was run in a separate lane (stained with ethidium bromide) and used as a size marker. Lane 1, CLL – 4 after incubation (22 hours) in the presence of PHA and TPA; lane 2, CLL – 4 after incubation (six hours) with PHA/TPA; lane 3, CLL – 4 after incubation (22 hours) without the addition of PHA/TPA; lane 4, CLL – 1, after incubation (22 hours) with PHA/TPA; lane 5, peripheral blood T lymphocytes after incubation (22 hours) with PHA/TPA; lane 6, PHA/TPA-activated (six hours) Jurkat cells; lane 7, Jurkat cells, 1/10 the amount of lane 6.**

culture media conditioned by these cells was not due to active production but represented an activity previously absorbed by the CLL cells from an exogenous source. On the other hand, it remains possible that the CLL cells did synthesize small quantities of IL2 but that the amount of IL2 mRNA remained below the detection level of the Northern blot analysis.

DISCUSSION

Recently it was shown that IL2 is a growth stimulator of B lymphocytes, and detailed information with respect to the responsiveness of B cells to IL2, their requirements of activation for the expression of membrane receptors for IL2, and the binding properties of the IL2 receptors has become available.[1-10] Similar studies carried out with leukemic cells should reveal whether the response of the cells to growth and differentiation stimuli has been altered as a result of neoplastic transformation. The present experiments were undertaken to determine the properties of IL2 receptors and the IL2 responsiveness of leukemic B lymphocytes, ie, in four cases of B CLL, and to establish whether these features were different from those reported for normal B lymphocytes.

From radiolabeled IL2 binding assays it became apparent that activated CLL cells express two classes of IL2 receptors, ie, with a high (picomolar range) affinity and with a low (nanomolar range) affinity (Table 2). Comparable data have been reported for murine B cell blasts.[5] Mean densities of IL2 receptors per cell on the mouse blast cells appeared to be considerably (about 200 times) higher than those on human B CLL cells. On the other hand, Muraguchi et al, without discriminating between high- and low-affinity binding sites, estimated the mean numbers of IL2 receptors per normal activated human B cell at 320 (with a mean Kd of 457 pmol/L).[4] The low mean numbers of high-affinity IL2-binding sites per cell that we observed in CLL (ie, ranging from 11 to 90) could indicate that these functional receptors are not equally distributed over the total CLL cell population but concentrated on the IL2-responsive clonogenic subfraction that comprises only a small minority (less than 0.1%) of the total CLL cell population.[14] It should be emphasized that the cell suspensions had not been enriched for blast cells as was described by Robb et al.[28] This may also explain why the mean numbers per cell of high-affinity IL2 receptors on normal activated T lymphocytes that we observed were ten to 20 times less than those reported by Robb et al for density gradient purified T blasts.[28]

In three cases (CLL-1, -2, and -3), proliferation as measured by ^{3}H-TdR incorporation was stimulated by IL2. The optimal IL2 concentration in these cases was 50 to 100 U/mL (Fig 4), ie, in the same order as reported for proliferation of normal B lymphocytes.[4,10] For an optimal response to IL2 the cells of CLL-1, -2, and -3 also required activation by PHA. This lectin has been previously shown to activate CLL cells.[14,30,31] In separate experiments not reported here, the phorbol ester TPA was used as the activation signal with comparable results.

In contrast to the earlier described response pattern of CLL-1, -2, and -3, the cells of CLL-4 proliferated independently on exogenous IL2 and did not require PHA as an activating factor (Figs 3 and 4). Nonetheless, after in vitro incubation the CLL-4 cells expressed functional IL2 receptors with a high affinity for IL2 (Table 2). Upon further analysis it became evident that spontaneous proliferation of the CLL-4 cells was most likely induced by endogenous IL2: specific blocking of IL2 receptors with monoclonal antibodies almost completely abrogated cell proliferation (Table 3). Moreover, concentrations of IL2 activity within the picomolar range were detected in the growth medium in which the cells from CLL-4 had been cultured. One could argue that this endogenous IL2 activity had been produced by activated contaminant T lymphocytes. For two reasons we consider this possibility unlikely: (a) In the fresh (T cell–depleted) material no T lymphocytes were detectable, and immunologic and cytogenetic analysis indicated that T lymphocytic cells had not expanded during culture either; (b) T lymphocytes require activation (eg, by the lectin PHA) or a phorbol ester for IL2 production, whereas spontaneous proliferation of the CLL-4 cells and elaboration of IL2 into the culture medium occurred in the absence of PHA. Therefore it is most plausible to assume that indeed the CLL cells (CLL-4) elaborated IL2 in the culture medium. The suggestion that normal and transformed B cells can produce cytokines, including IL2, was recently raised by several investigators.[32-34] To investigate whether the cells of CLL-4 were able to synthesize IL2 we attempted to detect IL2 mRNA in the cells, with, however, negative results (Fig 5). Thus, if the cells really produced IL2, they formed IL2 mRNA in a quantity below the detection level of the Northern analysis. Typically, this could be the case when only a small portion of the CLL cells, eg, the proliferating subpopulation under the applied in vitro conditions, synthesized IL2. More sensitive detection methods, eg, S1 nuclease mapping or in situ hybridization techniques, will be needed to clarify this issue. Other explanations for the failure to detect IL2 mRNA are that the cells of CLL-4 produced an IL2-like activity with a protein structure discrepant from T cell–derived IL2 or that the CLL cells released IL2 that had previously been absorbed by the cells from external sources.

Taken together, the present experiments have established that the proliferation of CLL cells in vitro is regulated by IL2 through direct IL2–IL2 receptor interaction, similar to that of normal B lymphoblasts. In addition, the experimental data could suggest that in some cases the CLL cells are capable of autocrine IL2-mediated stimulation. Autocrine growth may be the result of neoplastic transformation, and it has been suggested as a mechanism of maintaining tumor cell proliferation.[35] On the other hand, it has become clear that normal cells may be controlled by autocrine mechanisms of growth as well. For example, T lymphocytes proliferate in response to endogenous IL2.[36] It is unknown whether (subpopulations of) normal B cells may also be capable of autostimulation. Therefore, at the present time, it remains uncertain as to whether the growth characteristics of the cells of CLL-4 represent those of a normal B lymphocytic subset or whether they are typical of the transformed status of the neoplastic cells. Irrespective of this question, it is intriguing that primary tumor cells from individual patients that apparently descended from the same type of cell (ie, early B lymphocyte) may show markedly different in vitro growth characteristics.

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γ-Interferon Alters Globin Gene Expression in Neonatal and Adult Erythroid Cells

By Barbara A. Miller, Susan P. Perrine, Giovanna Antognetti, David H. Perlmutter, Stephen G. Emerson, Colin Sieff, and Douglas V. Faller

Interferons have the ability to enhance or diminish the expression of specific genes and have been shown to affect the proliferation of certain cells. Here, the effect of γ-interferon on fetal hemoglobin synthesis by purified cord blood, fetal liver, and adult bone marrow erythroid progenitors was studied with a radioligand assay to measure hemoglobin production by BFU-E–derived erythroblasts. Coculture with recombinant γ-interferon resulted in a significant and dose-dependent decrease in fetal hemoglobin production by neonatal and adult, but not fetal, BFU-E–derived erythroblasts. Accumulation of fetal hemoglobin by cord blood BFU-E–derived erythroblasts decreased up to 38.1% of control cultures (erythropoietin only). Synthesis of both $^G\gamma/^A\gamma$ globin was decreased, since the $^G\gamma/^A\gamma$ ratio was unchanged. Picograms fetal hemoglobin per cell was decreased by γ-interferon addition, but picograms total hemoglobin was unchanged, demonstrating that a reciprocal increase in β-globin production occurred in cultures treated with γ-interferon. No toxic effect of γ-interferon on colony growth was noted. The addition of γ-interferon to cultures resulted in a decrease in the percentage of HbF produced by adult BFU-E–derived cells to 45.6% of control. Fetal hemoglobin production by cord blood, fetal liver, and adult bone marrow erythroid progenitors, was not significantly affected by the addition of recombinant GM-CSF, recombinant interleukin 1 (IL-1), recombinant IL-2, or recombinant α-interferon. Although fetal progenitor cells appear unable to alter their fetal hemoglobin program in response to any of the growth factors added here, the interaction of neonatal and adult erythroid progenitors with γ-interferon results in an altered expression of globin genes. This supports the concept that developmental globin gene switching can be regulated by environmental factors.

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THE DEVELOPMENTAL SWITCH from γ to β globin chain production has been studied extensively as a model of gene regulation. An understanding of the controlling elements of this switch has therapeutic implications, since continued synthesis of fetal hemoglobin (HbF) into adult life could ameliorate the clinical manifestations associated with hemoglobinopathies, including sickle cell disease and β-thalassemia.[1]

HbF production by progenitor-derived erythroblasts has therefore been studied to determine factors involved in the control of γ globin and β globin gene expression. For example, the presence of adherent cells may increase the relative amount of HbF produced by adult progenitors.[2] Supernatant from Mo cells, a human lymphoid tumor line, has been found to enhance hemoglobin A synthesis by cord blood BFU-E–derived erythroblasts.[3] In addition, an uncharacterized factor in fetal sheep serum has been shown to modulate HbF production in cultures of neonatal and adult BFU-E and of BFU-E from patients with homozygous β-thalassemia and hereditary persistence of fetal hemoglobin (HPFH).[4,5] These observations suggested that soluble factors elaborated by cells are capable of influencing the type of globin gene expressed in erythroid progenitors.

Here, the effects of recombinant growth factors, particularly those elaborated by lymphoid cells like Mo, on HbF production by purified adult, cord blood, and fetal progenitors were studied to determine whether any of these factors are capable of modulating hemoglobin production. Partially purified cord blood progenitor cells were cultured with recombinant interleukin 1 (IL-1), IL-2, γ-interferon, or GM-CSF, and HbF production by BFU-E–derived erythroblasts was assessed by a radioligand assay. The addition of γ-interferon resulted in a significant decrease in HbF production by cord blood BFU-E–derived erythroblasts to 38.1% ± 2.3% of control, but total hemoglobin production was unaffected. Interferon also decreased the amount of HbF produced by adult progenitor–derived erythroblasts but not those derived from fetal liver. This pattern of modulation of globin expression is similar to that described in fetal sheep sera.[4] Recombinant γ-interferon specifically interacts with neonatal and adult progenitor cells by altering globin gene expression from predominantly γ to β production.

MATERIALS AND METHODS

Cord blood, bone marrow, and fetal liver samples. Cord blood samples were obtained from normal full-term newborn deliveries, and fetal liver samples were obtained at 18 to 21 weeks' gestation under a protocol approved by The Brigham and Women's Hospital Human Investigation Committee and The Milton S. Hershey Medical Center Committee on Clinical Investigation. Bone marrow samples were procured from normal volunteer donors after informed consent obtained within institutional guidelines.

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Purification of erythroid progenitor cells. Cord blood and bone marrow mononuclear cells were separated on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), centrifuged, and washed as previously described.[6] Fetal liver was treated with 50 mg/dL collagenase, type IV (Sigma Chemical Co, St Louis), and centrifuged over Ficoll-Paque twice as previously described.[7] Adherent cells from all samples were depleted by overnight adherence to 100 × 15-mm plastic culture dishes in Iscove's modified Dulbecco's medium (IMDM) with 20% fetal calf serum at 37°C. Nonadherent cells were then labeled for one hour at 4°C with optimal concentrations of antibodies that recognized precursors and mature myeloid and lymphoid cells to select for antigen-negative stem cells. Antibodies used to label cord blood cells included anti-Leu-1, -5b, -9, -10, -12, -M1, -M2, and -M3 (Becton Dickinson, Oxnard, CA); TG-1 (gift of Peter Beverley); and 10 F7 (gift of Dr William Bigbee). Antibodies to label adult bone marrow cells included anti-Leu-1, anti-Leu-5b, Campath 1, TG-1, anti-Leu-M1, MO1 (Ortho Pharmaceutical Corp, Raritan NJ), My-8 (gift of Dr J. Griffin), YTH 89.18 (gift of Dr Herman Waldman), and 10 F7[6]; antibodies to label fetal liver cells included anti-Leu-1, anti-Leu-5b, anti-Leu-10, anti-Leu-M1, anti-BA-1 (Hybritech, San Diego) anti-Calla (Becton Dickinson), TG-1, and 10 F7.[7] Mononuclear cells were washed to remove excess antibody and then incubated for one hour at 4°C on Petri dishes coated with affinity-purified rabbit antimouse IgG, IgA, and IgM. Nonadherent antibody-negative cells were removed by swirling the plates gently. The frequency of cord blood BFU-E present in nonadherent cells from this purification step was 1% to 4%, that of bone marrow BFU-E was 1%, and that of fetal liver BFU-E was 1% after adherence to plastic alone and up to 30% after panning. The final fraction cultured contained fewer than 1% lymphocytes and 0% to 5% monocytes.

Analysis of hemoglobin production by radioligand assay. Partially purified mononuclear cells from cord blood, bone marrow, or fetal liver were cultured in 0.9% methylcellulose media containing 30% fetal calf serum, 9.0 mg/mL deionized bovine serum albumin (fraction V, Sigma), 1.4×10^{-4} mol/L β-mercaptoethanol, and 2 μ/mL erythropoietin (Step I, Terry Fox Laboratories, Vancouver, Canada, a source without detectable burst-promoting activity). Recombinant generated human pI 7 IL-1 (gift of Cistron Technology, Pinebrook, NJ), recombinant IL-2 (gift of Biogen, Cambridge, MA), recombinant γ-interferon (gift of Biogen), recombinant α-interferon (gift of Schering Corp, Bloomfield, NJ), recombinant GM-CSF (Genetics Institute, Cambridge, MA), or Mo cell-conditioned medium (Mo-CM, provided by Dr David Golde) was also added where indicated. In some experiments, γ-interferon was added to culture dishes at day 0, 4, 6, 8, 10, or 12 of the culture period. Bone marrow and cord blood progenitors were plated at 5×10^3 cells/mL and fetal liver at 1.5×10^4 cells/mL (adherent cell depleted) or 5×10^3 cells/mL (panned). Cultures were performed in humidified 4% CO_2 at 37°C for 14 days, and erythroid colonies were then counted.

Approximately 50 to 100 BFU-E-derived colonies were plucked per sample and the average number of erythroid cells per colony determined. When samples were plucked in duplicate, the number of cells per colony differed from the average count by ±5% to 20%. A radioligand assay of total hemoglobin or HbF in picograms per BFU-E-derived cell was performed as previously described.[8-10] Calculations of hemoglobin content per BFU-E-derived cell were based on the assumption that all such cells are potential F cells. The percent HbF was calculated from picograms of HbF and the total hemoglobin cell. Reproducibility of this assay has been discussed previously,[10] but in general, duplicate dishes or multiple assays performed on samples from one individual on different days yielded results within 10% of the mean.

Analysis of hemoglobin accumulation, $^G\gamma/^A\gamma$ ratio, and globin synthetic ratios in BFU-E-derived erythroblasts by urea Triton X-100 gel. Hemolysates from BFU-E-derived erythroblasts were prepared. The ratio of γ to β chain accumulation in these erythroblasts and the $^G\gamma/^A\gamma$ ratio with and without the addition of γ-interferon to cultures was determined by urea-Triton X-100 gel electrophoresis as previously described.[11] Assessment of hemoglobin synthesis was performed by incubating plucked 13-day-old BFU-E-derived cells with 50 μCi ^3H-leucine-free minimum essential medium with 10% dialyzed fetal calf serum overnight, electrophoresis on Triton-urea gels, and autoradiography. Globin synthetic ratios were quantitated by densitometry.

RESULTS

Effect of recombinant growth factors GM-CSF, IL-1, IL-2, γ-interferon, and α-interferon on HbF synthesis by cord blood BFU-E-derived erythroblasts. Cord blood mononuclear cells were cultured in methylcellulose with and without Mo-CM to determine whether the addition of this source of burst-promoting activity had an effect on the quantity of HbF produced per BFU-E-derived erythroblast. In three out of four cord blood samples, the percentage of HbF measured per BFU-E derived erythroblast decreased in the presence of Mo-CM (Fig 1).

Further experiments were performed to determine which factor in Mo-CM was responsible for decreased HbF synthesis. Cord blood BFU-E were enriched in these experiments so that factors added would interact directly with progenitors rather than with contaminating T cells or macrophages. Either 5% Mo-CM, 5% recombinant GM-CSF from COS cell supernatant (100 μg/mL, 1/100 dilution), recombinant IL-1 (700 ng/mL final concentration), recombinant IL-2 (10 U/mL final concentration), recombinant γ-interferon (200 U/mL final concentration) or α-interferon (2,000 U/mL final concentration) was added to methylcellulose cultures on day 0. The concentration of GM-CSF was maximal

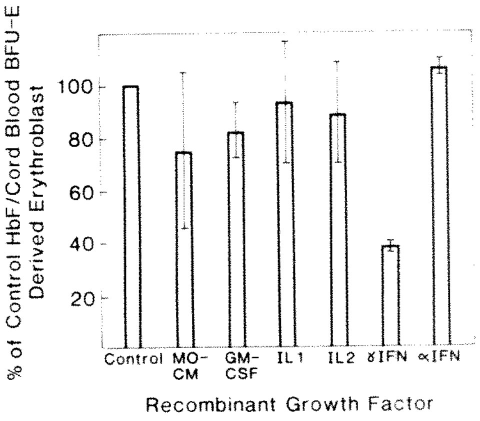

Fig 1. **Addition of 5% Mo-CM, 5% recombinant GM-CSF (100 μg/mL, 1/100 dilution), recombinant IL-1 (700 ng/mL), recombinant IL-2 (10 U/mL), recombinant γ-interferon (200 U/mL) or α-interferon (2000 U/mL) to methylcellulose cultures of purified cord blood BFU-E plated at 5×10^3 cells/mL. HbF content of 14-day-old BFU-E-derived erythroblasts was measured by a radioligand assay. Results are expressed as a percentage of control HbF (cultured with erythropoietin only), and the mean ±1 SD of four experiments is shown.**

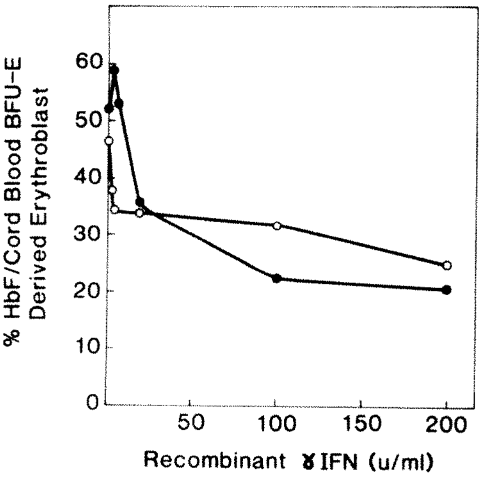

Fig 2. Addition of 0 to 200 U/mL recombinant γ-interferon added to cultures of purified cord blood BFU-E. The percentage of HbF/BFU-E–derived erythroblast was calculated from a radioligand assay for fetal and total hemoglobin.

for stimulation of erythroid colony growth. IL-1 was used at two different concentrations, 700 ng/mL and 2800 ng/mL, both at plateau concentrations for the stimulation of IL-1–dependent cells. Results were identical, and data are shown for the addition of 700 ng/mL. The concentration of IL-2 was on the plateau for support of IL-2–dependent cells, and γ-interferon (at 200 U/mL) was added at five times the amount required for maximal induction of class I major histocompatibility complex proteins on K562 cells. Results of experiments on four cord blood samples are summarized in Fig 1. To allow for comparison of four separate experiments, the percentage of HbF measured in all experimental groups was expressed as a percentage of the control (erythropoietin only). Mo-CM in the culture medium decreased HbF production to 75% of control, but the standard deviation was large. The addition of GM-CSF, IL-1, IL-2, or α-interferon to cultures resulted in the production of HbF that was not significantly different from the control. However, the percentage of HbF measured in BFU-E–derived erythroblasts incubated with γ-interferon was reduced to 38.1% ± 2.3% of control, a significant reduction.

To determine whether this effect was dose related, recombinant γ-interferon at 0 to 200 U/mL was added to methylcellulose cultures of partially purified progenitors. Results of two experiments are shown in Fig 2. The dose response to γ-interferon was greatest in the range of 0 to 20 U/mL, and maximal effect was nearly achieved at 100 U/mL.

The number of BFU-E–derived colonies and the number of cells/BFU-E–derived colony were determined in experiments to which recombinant growth factors were added. Results of three experiments are shown on Table 1. None of these growth factors affected progenitor proliferation at the concentration at which they were used here.

The picograms of HbF and total hemoglobin measured by radioligand assay of lysates from BFU-E–derived erythroblasts cultured with and without γ-interferon are shown for experiments on three cord blood samples in Table 2. Although the quantity of HbF in picograms per cell decreased with the addition of γ-interferon, no change in total hemoglobin content per cell was noted, which indicated a reciprocal increase occurred in the amount of β globin produced as the amount of γ globin decreased.

Effect of γ-interferon on globin synthesis by cord blood BFU-E–derived erythroblasts measured by ^{3}H-leucine incorporation. Additional experiments were performed to determine whether the effect of γ-interferon on the synthesis of HbF by BFU-E–derived erythroblasts occurred throughout colony maturation and hemoglobin synthesis. Partially purified cord blood BFU-E were cultured for 14 days with and without the addition of 200 U/mL γ-interferon added at day 0. The percentage of HbF produced over the entire culture period was measured by radioligand assay and by globin chain electrophoresis, and the percentage of γ chains synthesized on days 13 and 14 was measured by ^{3}H-leucine incorporation.

Results of three experiments are shown in Table 3. A decrease in γ globin accumulation for the 14-day culture period was noted after γ-interferon addition by measurement with both urea–Triton X-100 gel electrophoresis and radioligand assay. The mean percentage of HbF after γ-interferon addition on day 0, measured by radioligand assay, was 60.6% ± 2.9% of control cultures, less of an effect than noted in initial experiments. These differences may result from differences in individual cord blood samples or different γ-interferon lots. A marked decrease in γ globin synthesis was also noted by measurement of ^{3}H-leucine incorporation by BFU-E erythroblasts on days 13 and 14 of culture. The

Table 1. Effect of Recombinant Growth Factors on Cord Blood BFU-E–Derived Colony Growth

	Exp 1		Exp 2		Exp 3	
Factor	BFU-E*	Cells/ BFU-E†	BFU-E*	Cells/ BFU-E†	BFU-E*	Cells/ BFU-E†
IMDM Control	232 ± 3	8.7	47 ± 4	2.5	104 ± 4	7.2
Mo-CM	290 ± 9	5.3	50 ± 5	6.8	120 ± 12	4.3
GM-CSF	250 ± 9	8.9	50 ± 3	3.5	121 ± 9	6.6
IL-1, 700 ng/mL	274 ± 25	9.3	44 ± 10	3.7	136 ± 8	6.6
IL-2, 20 U/mL	272 ± 30	9.6	48 ± 7	2.6	83 ± 3	8.5
γIFN, 200 U/mL	262 ± 12	6.8	63 ± 14	2.0	110 ± 16	8.0

Abbreviations: Exp, experiment; γIFN, γ-interferon.

*Mean number ± 1 SD of BFU-E–derived colonies/5 × 10^3 enriched cord blood mononuclear cells.

†Number of cells × 10^3/BFU-E–derived colony.

Table 2. Effect of γIFN on Fetal and Total Hemoglobin Accumulation in Cord Blood BFU-E-Derived Erythroblasts

	HbF/Cell (pg)	Total Hb/Cell (pg)	Percentage of HbF/Cell	Percentage of Control
Exp 1				
Control	10.5	31.1	33.8	
γIFN	4.57	34.4	13.3	39.3
Exp 2				
Control	29.5	43.3	68.2	
γIFN	18.9	69.9	26.9	39.4
Exp 3				
Control	16.69	32.2	51.8	
γIFN	7.98	39.0	20.5	39.6

Results from three different cord blood samples are shown. Control cultures contained erythropoietin only. A quantity of 200 U/mL γIFN was added to the experimental group. Picograms fetal and total hemoglobin were measured by radioligand assay of lysates from 14-day-old BFU-E-derived erythroblasts.

results of one experiment measuring ^{3}H-leucine incorporation by BFU-E-derived erythroblasts with and without incubation with γ-interferon is shown in Fig 3.

The $^G\gamma/^A\gamma$ ratio was measured in two of these experiments by globin chain electrophoresis. The $^G\gamma/^A\gamma$ was 69.5% (cord blood sample 1) and 61.0% (cord blood sample 2) in control studies and 67.7% (sample 1) and 65.6% (sample 2) with coculture of BFU-E with γ-interferon.

Addition of γ-interferon to cord blood BFU-E cultures at various times during incubation. γ-Interferon at 200 U/mL was added to cultures of cord blood BFU-E on day 0, 4, 6, 8, 10, or 12. BFU-E-derived erythroblasts were harvested on day 14, and a radioligand assay was performed to determine the quantity of HbF produced. Results are shown in Fig 4. A marked decrease in HbF accumulation was noted when γ-interferon was added on day 0, 4, or 6. This effect on HbF accumulation decreased dramatically by day 10, probably because the majority of HbF production had already occurred in progenitor-derived cells at that time.[12] After day 12, effects of addition of γ-interferon on HbF production could no longer be detected by measurement of accumulation.

Effect of γ-interferon on HbF production by individual cord blood BFU-E-derived colonies. It is possible that γ-interferon inhibits the growth of a hypothetical "fetal" BFU-E population. To test this possibility, partially purified cord blood BFU-E were cultured with and without γ-interferon. On day 14 of culture, individual BFU-E from both groups were plucked, and the hemoglobin content was assessed by radioligand assay. Results are shown in Fig 5. When γ-interferon was added to the cultures, HbF production was reduced from a mean ± 1 SD of 68.5% ± 25.5% to 35.8% ± 21.2% ($P < .0001$). Few γ-interferon-treated BFU-E-derived colonies produced >50% γ-globin, and many produced <25% γ globin. No such BFU-E populations

Table 3. Comparison of HbF Synthesis/Cord Blood BFU-E-Derived Erythroblasts by Different Methods

Method	Percentage of HbF or γ Globin/BFU-E-Derived Cells Cord Blood 1	Cord Blood 2	Cord Blood 3
Radioligand Assay*			
Control	72.0	47.0	79.0
+γIFN	44.0	30.0	44.9
Accumulation by urea-Triton gel†			
Control	57.0	40.0	—
+γIFN	36.0	30.0	—
^{3}H-leucine synthesis‡			
Control	53.3	42.5	39
+γIFN	38.6	29.6	0

*The percentage of HbF is calculated from the measurement of fetal and total hemoglobin present in BFU-E-derived erythroblasts by radioligand assay.

†The percentage of γ globin is calculated from the measurement on a urea-Triton X-100 gel of γ and β globin chains synthesized during a 14-day culture of BFU-E-derived erythroblasts.

‡The percentage of γ globin is calculated from the measurement of ^{3}H-leucine incorporation by γ and β globin chains synthesized during an overnight incubation of day-13 BFU-E-derived erythroblasts, a time during colony maturation when β globin synthesis is normally predominant.

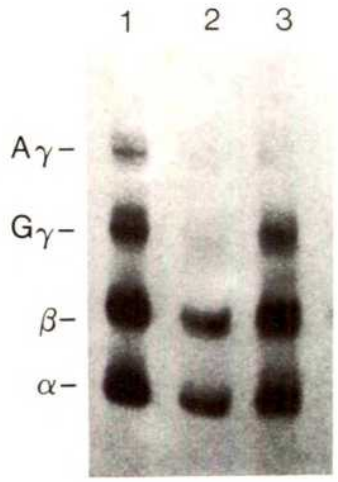

Fig 3. Autoradiograph of globin produced by cord blood BFU-E. Lanes 1 and 3 represent BFU-E cultured without γ-interferon; lane 2 shows BFU-E from the same cord blood sample cultured with γ-interferon.

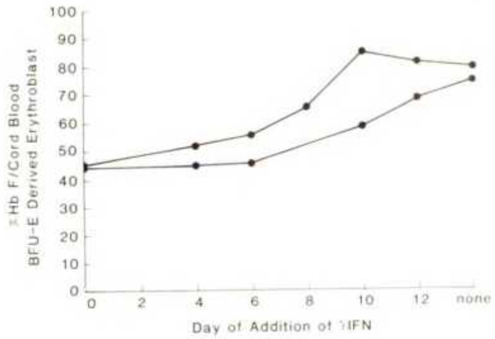

Fig 4. Assessment of the percentage of HbF per cord blood BFU-E-derived cell after the addition of 200 U/mL γ-interferon to cultures on day 0, 4, 6, 8, 10, or 12. BFU-E-derived colonies were harvested on day 14 and the percentage of HbF measured by radioligand assay.

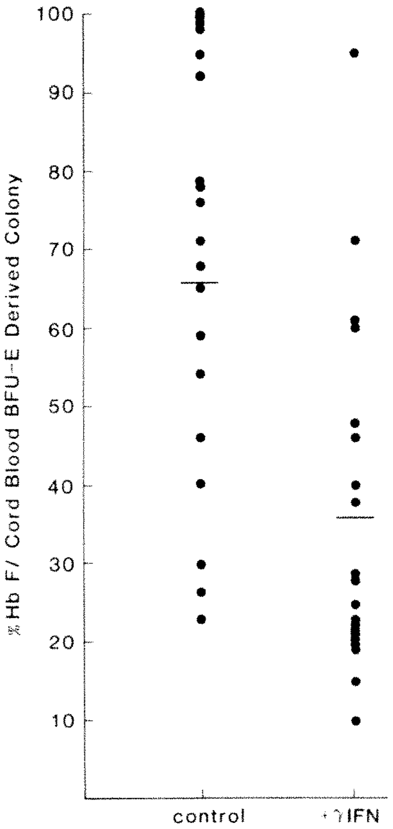

Fig 5. The percentage of HbF is measured in individual cord blood BFU-E–derived colonies cultured with erythropoietin only (control) or with 200 U/mL γ-interferon. BFU-E–derived colonies were harvested on day 14 and the percentage of HbF measured by radioligand assay.

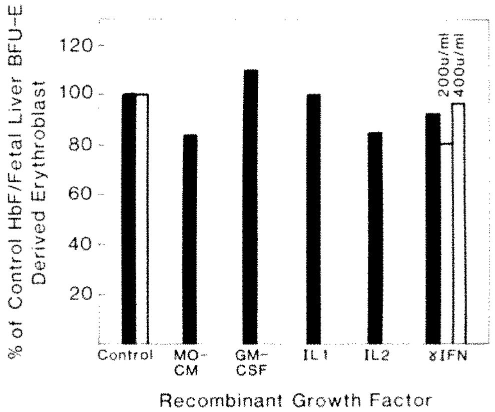

Fig 6. Addition of 5% Mo-CM, 5% recombinant GM-CSF (100 μg/mL, 1/100 dilution), recombinant IL-1 (700 ng/mL), recombinant IL-2 (10 U/mL), and recombinant γ-interferon (200 U/mL or 400 U/mL) to cultures of adherence-depleted (■, two experiments) or -purified (☐, one experiment) fetal liver BFU-E. The HbF content of BFU-E–derived erythroblasts was measured by radioligand assay. Results are expressed as a percentage of the control (erythropoietin only) HbF. An average of results for two experiments with adherence-depleted progenitors is shown.

producing <23% γ globin were seen in the absence of γ-interferon. These results suggest a decrease in HbF production by all BFU-E–derived colonies.

Effect of recombinant growth factors GM-CSF, IL-1, IL-2, and γ-interferon on hemoglobin synthesis by fetal liver BFU-E–derived erythroblasts. Fetal liver mononuclear cells prepared by adherence depletion of macrophages (two samples) or by partial purification (panning, one sample) were cultured with and without the addition of 5% Mo-CM, 5% recombinant GM-CSF (100 μg/mL, 1/100 dilution), recombinant IL-1 (700 ng/mL), recombinant IL-2 (10 U/mL final concentration), or recombinant γ-interferon (200 or 400 U/mL). BFU-E–derived erythroblasts were harvested on day 14 and the hemoglobin content determined by radioligand assay. Results are shown in Fig 6. None of these factors significantly affected HbF production from fetal liver progenitors. When γ-interferon was added in a range of 0.2 to 400 U/mL, no effect was seen (data not shown). Doses of 200 U/mL or 400 U/mL γ-interferon, which produced a significant effect on HbF accumulation in cord blood BFU-E–derived cells, had no effect on HbF production by fetal liver.

Effect of recombinant growth factors GM-CSF, IL-1, IL-2, and γ-interferon on hemoglobin synthesis by adult BFU-E–derived erythroblasts. Partially purified BFU-E from normal adult bone marrow were prepared as described earlier and were cultured with 5% Mo-CM, 5% recombinant GM-CSF (100 μg/mL, 1/100 dilution), recombinant IL-1 (700 ng/mL, final concentration), recombinant IL-2 (10 U/mL, final concentration), or γ-interferon (200 U/mL). The number of BFU-E–derived colonies and the number of cells per colony were reduced in adult samples without the addition of burst-promoting activity (present in Mo-CM and GM-CSF). Therefore, both GM-CSF and γ-interferon were added to some of the cultures where indicated. BFU-E–derived erythroblasts were plucked on day 14 and the hemoglobin content assessed by radioligand assay. Results are shown in Fig 7. The addition of γ-interferon significantly decreased the quantity of HbF produced in cultures of adult BFU-E–derived erythroblasts, as did the addition of both γ-interferon and GM-CSF ($P < .005$ compared with GM-CSF alone, calculated by one-way analysis of the variance). The addition of IL-2 resulted in a slight but not significant decrease in HbF synthesis. This may have resulted from γ-interferon production by residual T cells stimulated by IL-2. The group to which IL-1 was added was not significantly different ($P > .05$) in HbF production from those to which Mo-CM or GM-CSF was added.

DISCUSSION

The mechanisms controlling the fetal globin switch and γ and β globin gene regulation remain poorly understood. Analysis of DNA sequences in the γ region of patients with HPFH provides strong evidence that promoter regions exist in the first 200 base pairs, 5′ to the γ globin gene,[13-16] and experiments with human hybrid globin genes in mouse erythroleukemia cells (MEL cells) suggest that DNA sequences regulating β globin gene expression are located 5′

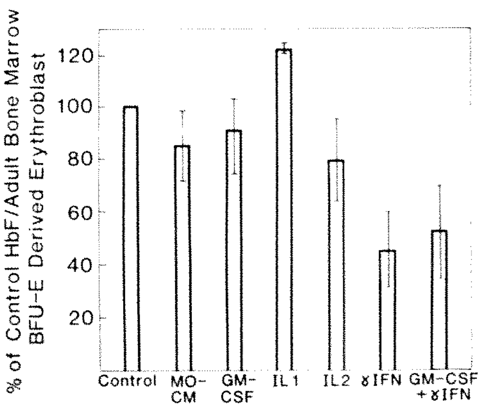

Fig 7. Addition of 5% Mo-CM, 5% recombinant GM-CSF (100 μg/mL, 1/100 dilution), recombinant IL-1 (700 ng/mL), recombinant IL-2 (10 U/mL), recombinant γ-interferon (200 U/mL), or GM-CSF plus γ-interferon to cultures of purified adult bone marrow BFU-E. The HbF content was measured by radioligand assay. Results are expressed as a percentage of the control (erythropoietin only) HbF. The mean ±1 SD of five experiments is shown.

and 3′ of the transcription initiation site.[17,18] The evidence for *trans*- and *cis*-acting factors that interact with these promoter regions has recently been reviewed,[19,20] but none of these factors has yet been identified.

Several in vivo transplantation experiments suggest that hemoglobin switching is largely controlled by mechanisms intrinsic to the stem cell.[21-23] Other in vivo studies demonstrate environmental influences do play a role. For example, transplantation of fetal cells into adult sheep results in synthesis of adult hemoglobin.[24] The γ-to-β globin switch is delayed in 80% of infants of diabetic mothers.[25] Several factors, including erythroid potentiating activity[3] and factors produced by adherent cells,[2] can affect HbF synthesis in culture. A factor present in fetal sheep sera was found to inhibit HbF synthesis in cultures of BFU-E from β^+-thalassemia patients,[4] HPFH patients,[5] normal adults, and neonatal blood[4] but had no effect on fetal liver progenitors.[4] The γ-to-β globin gene switch in BFU-E–derived erythroblasts from infants of diabetic mothers and premature infants is inhibited by the addition of sodium butyrate to cultures.[26] These observations show that interactions between progenitors and the inductive environment can affect HbF synthesis.

Here, we confirm earlier reports that a factor in Mo-CM has the ability to reduce HbF synthesis in culture of BFU-E–derived erythroblasts. We tested the recombinant growth factors GM-CSF, IL-1, IL-2, γ-interferon, and α-interferon to determine whether one of these cytokines was responsible. Recombinant γ-interferon significantly decreased HbF production in cord blood BFU-E–derived erythroblasts in a dose-dependent manner. This decrease resulted from an absolute decrease in HbF production and a reciprocal increase in adult hemoglobin production. HbF production was most dramatically affected when γ-interferon was present early in the culture period (Fig 4), as would be expected since HbF is synthesized first in culture. However, even at 13 days of culture, HbF synthesis was reduced, as shown by ^{3}H-leucine incorporation studies (Table 3). Assays of individual BFU-E–derived colonies after γ-interferon addition suggest that γ-interferon decreases the ability of all progenitor-derived colonies to produce HbF (Fig 5). The possibility that a "fetal" population is suppressed by γ-interferon and an "adult" population recruited seems unlikely because after γ-interferon addition the number of BFU-E was unchanged and all BFU-E–derived colonies still produced greater than 10% HbF, which is above the normal adult range (<10% HbF).

These data suggest that γ-interferon may play a role in the fetal globin switch. γ-Interferon production has been shown to be decreased in neonatal cells relative to adult cells due to both a decreased intrinsic capacity for γ-interferon production and to differences in regulatory mechanisms.[27,28] Whether the increased ability to produce γ-interferon during maturation of the fetus correlates with the decrease in HbF synthesis is unknown. γ-Interferon had no effect on HbF production by BFU-E–derived erythroblasts from fetal liver. This suggests that a property intrinsic to the fetal liver BFU-E or cells derived from it determines the early HbF program. However, in the neonate and adult, progenitor-derived cells are now susceptible to environmental interactions such as those with γ-interferon, thereby leading to a decrease in HbF production. Because purity of the progenitor cells is not absolute, the possibility that γ-interferon acts indirectly through nonprogenitor cells, which differ in the fetus compared with the neonate and which are responsible for differences observed, could not be ruled out. The effects of γ-interferon on HbF production are remarkably similar to those of fetal sheep sera previously reported by Papayannopoulou et al.[4,5] Both decrease HbF production by neonatal and adult but not fetal BFU-E–derived cells, and both have an effect on $^{G}\gamma$ and $^{A}\gamma$ production.[5] Whether γ-interferon is partly or wholly responsible for the switching activity in fetal sheep sera is not clear but seems unlikely because interferons have been shown not to work across species barriers.[29]

The mechanism(s) by which γ-interferon decreases γ globin production is not yet clear. Interferons exert effects on cells by binding to specific high-affinity receptors located on the cell surface, and this interaction is followed by activation of certain genes and decreased expression of others.[30-32] It has recently been shown that endogenous interferon produced during the differentiation of mouse erythroleukemia cells is in part responsible for decreased *c-myc* RNA synthesis and the arrest of cell proliferation observed during differentiation.[32] Production of and response to endogenous interferon takes place during the colony stimulating factor (CSF)-1–induced differentiation of normal bone marrow precursors to macrophages.[32] A role of endogenous interferon in erythroid differentiation may also exist. Recombinant γ-interferon has been reported to inhibit the proliferation of hematopoietic progenitors in a dose-dependent manner in short-term and long-term cultures, although the plateau was reached at much higher concentrations than those used here.[33] Recombinant γ-interferon has also been reported to synergistically inhibit CFU-GM in combination with lymphotoxin.[34] The role of γ-interferon as a natural regulator of hematopoietic differentiation and HbF production in vivo requires further investigation, particularly into whether changes in chromatin structure are involved or required for this "environmental" effect.[35]

In summary, coculture with recombinant γ-interferon results in decreased HbF synthesis by neonatal and adult but not by fetal BFU-E–derived erythroblasts, and this decrease is dose dependent. Synthesis of $^{G}\gamma$, $^{A}\gamma$, and β globin is affected throughout the period of hemoglobin production. During late fetal development, erythroid progenitors appear to acquire the capacity to respond to factors in their environment by altering their pattern of globin gene expression.

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T Lymphocytes Lack Rearrangement of the *bcr* Gene in Philadelphia Chromosome–Positive Chronic Myelocytic Leukemia

By Claus R. Bartram, Anand Raghavachar, Bernd Anger, Christoph Stain, and Peter Bettelheim

To study the possible involvement of T lymphocytes in Philadelphia chromosome (Ph)-positive chronic myelocytic leukemia (CML) we analyzed the arrangement of the *bcr* gene in T cell and non-T cell samples of 12 CML patients. Although all the patients showed *bcr* rearrangements in non-T cell fractions, T cell populations lacked respective gene recombinations. Moreover, by Southern blot analyses using T cell receptor β chain sequences our data indicate polyclonality of T cell samples from 11 of 12 cases; in one patient a clonal T cell population could be identified. These results suggest that T lineages of most Ph-positive CML patients are not derived from pluripotent stem cells involved in leukemogenesis and thus confirm previous investigations based on cytogenetic or glucose-6-phosphate dehydrogenase analyses. The demonstration of polyclonal T cell populations may reflect persistence of stem cells committed to differentiate only into T cells.

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CHRONIC MYELOCYTIC LEUKEMIA (CML) originates in a pluripotent hematopoietic stem cell that gives rise to granulocytes, eosinophils, basophils, erythrocytes, megakaryocytes, monocyte/macrophage cells, and B lymphocytes.[1-3] This clonal nature of CML has been mainly established by cytogenetic and glucose-6-phosphate dehydrogenase (G6PD) isoenzyme amalyses.

The extent to which T lymphocytes are involved in the malignant process is still a matter of controversy. The majority of peripheral blood T cells responding to mitogen have not shown the Philadelphia (Ph) chromosome and are polyclonal by G6PD analyses.[4,5] On the other hand, rare reports on T cell blast crisis,[6-12] Ph-positive T cells obtained from multilineage colonies,[13] or clonal G6PD patterns in T cells of CML patients[14] indicate that the T lineage may be, albeit rarely, involved in Ph-positive CML.

In this report we addressed this topic by an alternative approach that is neither dependent on in vitro stimulation of respective cell populations nor restricted to the limited number of patients suitable for G6PD analyses. Proceeding from (1) the molecular hallmark of Ph-positive CML, ie, a rearrangement between *c-abl* and *bcr* genes,[15] and (2) T cell receptor sequences as a unique clonality marker[16] we investigated T cell and non-T cell fractions of 12 Ph-positive CML patients by Southern blot analysis.

MATERIALS AND METHODS

Patients. After informed consent was given peripheral blood samples were obtained from 12 Ph-positive CML patients. Some clinical data are listed in Table 1. Ten patients were in chronic phase at the time of study, from 1 to 59 months after diagnosis. Patient 4 had been in an accelerated state for 3 months. Cytogenetic analyses showed a standard translocation (9;22) in these 11 cases. Patient 11 developed lymphocytic blast crisis 3 weeks prior to the investigation, and a bone marrow specimen showed 50% blasts of common acute leukemia antigen (CALLA)+ phenotype and a genotype characterized by monoclonal Igcμ rearrangement as well as CTβ germline configuration; besides showing 40% of the metaphases exhibiting a t(9;22), 60% of the blasts revealed additional chromosome aberrations, ie, trisomies 8 and 12.

Isolation of cell samples. Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala Sweden). PBMC (1.2 to 1.8 × 10^7/mL) were mixed with an equal volume of neuraminidase-treated (0.2 U *Vibrio cholerae* neuraminidase/mL, Behringwerke, Marburg, FRG) sheep erythrocytes (5% in RPMI). Thereafter the cells were centrifuged on Ficoll-Paque for 30 minutes at 1,800 rpm at room temperature. Interphase cells were washed twice and applied as the non-T cell fraction in Southern blot analysis. Contamination of this cell fraction with T lymphocytes never exceeded 2% as confirmed by staining with monoclonal antibody (MoAb) T11 (Ortho Pharmaceutical, Raritan, NJ).

After lysis of rosetted sheep erythrocytes, T cells obtained from the pellet were analyzed for purity by T11 staining. In cases exhibiting <97% purity, cell fractions were incubated with MoAb VIM-D5 (20 μg/mL, kindly provided by W. Knapp, Vienna), for 45 minutes at 4°C and consequently exposed to undiluted rabbit complement (Behringwerke) for 90 minutes at 37°C. After this procedure, the purity of T cell samples exceeded 98% as confirmed by indirect immunofluorescence staining with MoAb T11.

Southern blot analysis. DNA was extracted from T and non-T cell populations by standard techniques. Ten micrograms of DNA was digested with appropriate restriction enzymes (Boehringer Mannheim, FRG), electrophoresed on a 0.7% agarose gel, and blotted and hybridized as described.[15] λ-DNAs were included as molecular weight standards (not shown). *Bgl* II-digested DNA was hybridized to a 2-kb *Bgl*II/*Hind*III 5′ *bcr* probe[17] that detects a 5-kb germline fragment, and *Eco*RI or *Bam*HI digests were hybridized to CTβ sequences[18] detecting 4.2- (Tβ2) and 12-kb (Tβ1) or 23 kb (Tβ1 + Tβ2) germline fragments, respectively. Filters were exposed to XAR-2 films (Kodak, Rochester, NY) for 12 hours at −70°C with intensifying screens.

RESULTS

Southern blot analyses revealed a rearrangement of the *bcr* gene in non-T cell populations of all Ph-positive CML patients (Fig 1, lane a). However, respective T cell fractions lacked rearranged *bcr* sequences (Fig 1, lane b). Long time exposures (up to 3 weeks) of the filters also failed to exhibit rearranged *bcr* bands in all T cell populations (not shown). Taking into account the detection limits of Southern blot

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Table 1. Clinical Data of CML Patients

No.	Sex	Age (yr)	Time After Diagnosis (mo)
1	M	56	10
2	M	44	4
3	F	29	1
4	F	54	69
5	F	58	4
6	M	53	1
7	F	79	1
8	F	68	59
9	F	22	5
10	F	57	10
11	F	59	9*
12	F	25	42

*In blast crisis.

analyses (~1% clonal cells) the presence of a Ph translocation is virtually ruled out for more than 99% of the T cells studied in each case. In case 11, a faint hybridization signal of the rearranged fragment in the T cell sample represents in all likelihood a contamination with non-T cells (3%) since these cells had not been lysed after E rosetting. Moreover, the alternative explanation that a minor subset of T cells is characterized by a *bcr* rearrangement appears to be less likely because Tβ gene analyses (see the following paragraphs) demonstrated a clonal relationship of all T cells.

Southern blots showed a germline configuration of T cell receptor β chain genes in all non-T samples, as expected (Fig 2, lane a). T cell populations of 11 cases exhibited only the 4.2-kb germline band, whereas the 12-kb fragment was absent. *Bam*HI digests failed to detect rearranged fragments (not shown). This configuration is observed in polyclonal T cell populations.[16,19] The reason for the loss of the 12-kb band in polyclonal T cells as compared with the 4.2-kb band is evident from an *Eco*RI restriction site just before Cβ2 sequences. Thus the site of the 4.2-kb *Eco*RI fragment bearing the Cβ2 gene does not move, even in the case of Cβ2 rearrangements.

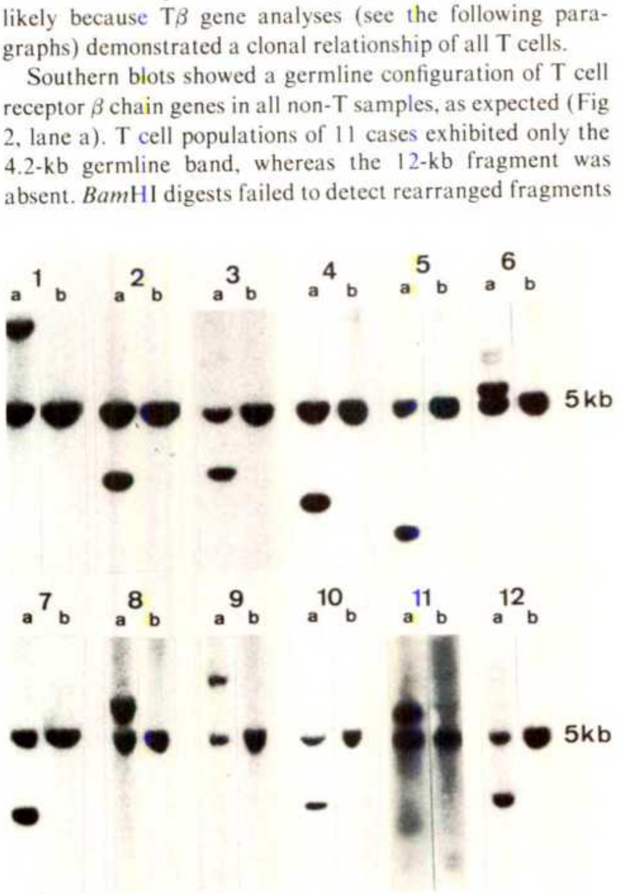

Fig 1. Southern blot analyses of non-T (a) and T cells (b) obtained from 12 Ph-positive CML patients. *Bgl*II digests (10 μg) were hybridized to a 5′ *bcr* probe detecting a 5-kb germline fragment.

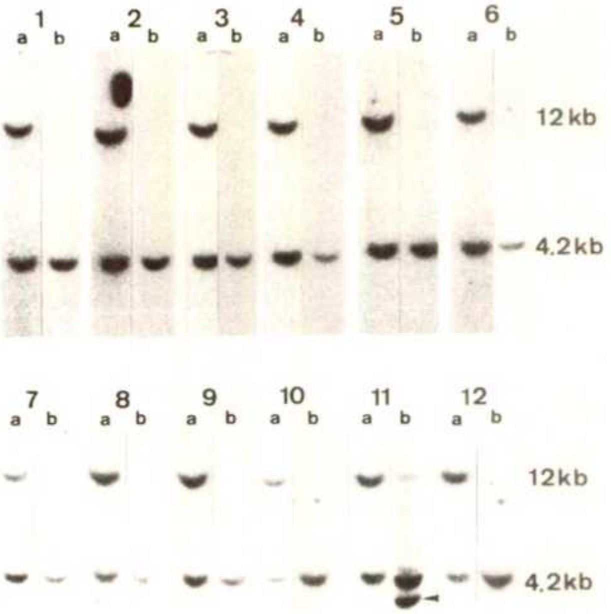

Fig 2. Southern blot analysis of non-T (a) and T cells (b) from 12 Ph-positive CML patients. *Eco*RI-digested DNAs (10 μg) were hybridized to CTβ sequences that detect 4.2-kb and 12-kb germline bands. Arrow points to a rearranged fragment in T cells from patient 11.

Surprisingly, a monoclonal Tβ gene rearrangement was observed in T cells of patient 11 (Fig 2, lane 11b); this result was confirmed for *Bam*HI digests (not shown). Leukemic blasts of this patient showed a B cell phenotype and genotype as well as the rearranged *bcr* fragment (Fig 1, lane 11a). On the contrary, T cells showed neither *bcr* nor IgCμ rearrangements. Moreover, the clonal T genotype contrasts with the phenotypic analysis of this T cell fraction that shows no restriction to a specific T cell subset (terminal deoxynucleotidyl transferase [TdT]$-$, T_3+, T_4+/T_8+ in normal ratio).

DISCUSSION

Ph-positive CML is molecularly characterized by a rearrangement of the *c-abl* oncogene and a gene provisionally called *bcr*.[15,20] As a consequence of this genomic recombination on the Ph chromosome, CML cells transcribe a chimeric 8.5-kb RNA species consisting of both 5′ *bcr* and *c-abl* sequences.[21,22] This molecule is translated into a p210 *c-abl* protein that differs from the normal p145 *abl* counterpart as to associated tyrosine kinase activity.[24]

In this study we used sequences of the *bcr* gene as a specific molecular marker for Ph-positive CML and investigated a possible involvement of T cells in leukemic cell populations. Our results indicate that T cells of all 12 patients lack Ph-specific *bcr* rearrangements. This is in agreement with previous reports based on G6PD or cytogenetic analyses.[4,5,14] However, since Southern blot analysis will only detect clonal populations of >1%, we cannot totally rule out the existence of minor, affected T cell clones.

Several observations suggest that the generation of Ph-positive CML is a multistep process in which a yet-unidentified first event causes the proliferation of pluripotent stem cells and a consecutive step is identified by genomic alterations associated with the Ph translocation.[25] To rule out the possibility that the T cell population of the 12 CML patients shows no *bcr* rearrangement but is nevertheless clonally related, we performed T cell receptor β chain analyses on respective samples. With case 11 being the only exception, all T cell fractions showed a polyclonal pattern.

The Tβ gene rearrangement of case 11 cannot readily be explained. The clonal genotype of these T cells, despite phenotypic heterogeneity, would suggest that an immature thymocyte, already characterized by a specific Tβ gene rearrangement, is nevertheless able to differentiate into different functional T cell subsets. From the molecular analyses reported here and in the absence of G6PD studies, we cannot settle the relationship between this T cell clone and the blast cell population. One possibility would be that both clones originated from a common, pluripotent stem cell. However, it is difficult to understand how, during the evolution of the primary stem cell clone, two different subclones developed and why the non-T clone led to the clinical manifestation of CML whereas the T cell clone appears to be biologically inert.

One possible explanation for the polyclonality of T lymphocytes in the majority of investigated cases could be that they are long-lived and antedate the development of CML. However, the fact that our studies were performed up to 5 years after the onset of the leukemia favors another view. Thus the demonstration of polyclonal, *bcr* rearrangement-negative T cells may reflect persistence of stem cells committed to differentiate only into T cells, as already suggested by Fialkow et al.[14]

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A Killing Defect of Natural Killer Cells With the Absence of Natural Killer Cytotoxic Factors in a Child With Hodgkin's Disease

By Atsushi Komiyama, Hiroshi Kawai, Sachihiro Yamada, Masao Kato, Mitsuhiko Yanagisawa, Yukiaki Miyagawa, and Taro Akabane

A killing defect of natural killer (NK) cells in the absence of NK cytotoxic factors (NKCF) was first demonstrated in a child with Hodgkin's disease. The patient lacked detectable NK cell activity in every phase of the disease as measured by a four-hour ^{51}Cr-release assay using K562 cells as a target. The percent lysis at a 40:1 effector:target ratio by the patient's lymphocytes was persistently below 0.3% as compared with the normal lymphocyte value of 46.2% ± 5.8% (mean ± SD). NK cell activity was not detectable at effector:target ratios of 10:1 to 80:1 and by prolongation of the incubation time, and the NK cell defect was not restored or improved by lymphocyte stimulation with polyinosinic-polycytidilic acid, interferon (IFN)-α, or interleukin 2 (IL 2). The numbers of Leu-7$^+$ cells and Leu-11$^+$ cells were normal as counted by flow cytometry. A single cell-in-agarose assay demonstrated normal numbers of target binding cells (TBCs), and they showed the morphology of "large granular lymphocytes." However, there were no TBCs with dead targets. These results indicated that the patient's lymphocytes contained normal numbers of NK cells that were capable of recognizing and binding to a target but were incapable of killing the bound target cell. The patient's lymphocytes were then studied for their release of NKCF upon interaction with K562 cells. The patient's cells did not release NKCF, and the NK cell defect was not restored or improved by stimulation of the cells with IFN or IL 2. It is suggested that the deficient release of NKCF may have been related to the killing defect of the NK cells in this patient.

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NATURAL KILLER (NK) cells are considered to be non-T, non-B lymphocytes[1] and play important roles in the defense against viral infections and tumor growth.[2,3] NK cells can lyse a target cell through recognition, binding, killing, and detaching, and they repeat this lytic sequence by the recycling capacity.[4,5] Soluble NK cytotoxic factors (NKCF)[6,7] and NK granule cytolysin,[8,9] which are released from NK cells after contact with NK-sensitive target cells, are known to be involved in the killing step.

A variety of functional defects have been documented in such leukocytes as neutrophils, monocytes/macrophages, T cells, and B cells,[10] but little is known about dysfunctions of NK cells.[11,12] Targan and Oseas[11] demonstrated a NK cell defect in Chédiak-Higashi syndrome. In this syndrome, a relative refractoriness in initiating the postbinding lytic mechanism leads to the lack of NK cell activity, which was termed lazy NK cells. We have recently demonstrated that there is impaired recycling of NK cells as a cause of depressed NK cell activity in some types of childhood chronic neutropenia.[12]

We studied a child with Hodgkin's disease who lacked detectable levels of NK cell activity for more than 4 years, and we analyzed the NK cell defect. In this article, we demonstrate a killing defect of NK cells in the absence of NKCF.

CASE REPORT

A 7-year-old boy presented in January 1982 with cervical lymphadenopathy. He had suffered from recurrent infections such as upper respiratory tract infections and otitis media since infancy. The family history was unremarkable.

At presentation, there were two enlarged lymph nodes measuring 2 cm × 2 cm × 2.5 cm in the left and in the right cervical region. The hemoglobin level was 13.8 g/dL; the platelet count 379×10^3 μL; and the leukocyte count 7,400/μL with 37% neutrophils, 3% eosinophils, 8% monocytes, and 52% lymphocytes. The bone marrow was normal. The serum IgM, IgG, and IgA levels were 57, 483, and 55 mg/dL, respectively. Delayed hypersensitivity against candidal antigen was positive. Liver and renal function test results were normal. Chest x-ray films, bipedal lymphangiography, echography, and computerized tomography did not show mediastinal and retroperitoneal lymphadenopathy. A garium scan demonstrated increased accumulation in the left cervical region. A specimen from excisional cervical lymph node biopsy revealed Hodgkin's disease of a mixed cellularity type.

On March 9, the patient was started on the first of six cycles of MOPP, which consisted of 6 mg/m^2 nitrogen mustard intravenously (IV) on days 1 and 8, 1.4 mg/m^2 vincristine IV on days 1 and 8, 100 mg/m^2 procarbazine orally daily for 14 days, and 40 mg/m^2 prednisolone orally daily for 14 days. Two days later, irradiation (4,000 rad in total) to the bilateral cervical regions was initiated. The cervical lymphadenopathy disappeared within 2 weeks after the institution of the treatments. The patient completed the therapy by the end of August 1982, and he has been in complete remission for more than 4 years. He is doing well except for frequent episodes of upper respiratory tract infections.

MATERIALS AND METHODS

Sample preparation. Heparinized peripheral blood samples were obtained from the patient before, during, and after therapy. Mononuclear cells were separated by Ficoll-Hypaque centrifugation from the blood samples, depleted of monocytes by adherence, and used as the patient's lymphocytes. For comparison, lymphocytes were obtained from six children with Hodgkin's disease ranging in age from 5 to 15 years; their clinical stages (An Arbor) were IA, IIA,

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or IIB, and the histologic subtypes (Rye) were lymphocytic predominance in four and mixed cellularity in two of them. Normal lymphocytes were also obtained, in an identical manner, from healthy volunteers.

For some experiments, lymphocytes (2 to 3 × 10^6/mL) were incubated with 30 μg/mL polyinosinic-polycytidilic acid (poly I:C; Calbiochem Behring, La Jolla, CA), 1,000 U/mL recombinant interferon (IFN)-α (Kyowa Hakko Co, Tokyo), or 1,000 U/mL recombinant interleukin 2 (IL 2) (Takeda Seiyaku Co, Osaka, Japan) at 37°C for 20 hours in a 5% CO_2 atmosphere. They were washed and used as effector cells.

For testing the inhibition of NK cells by serum, serum samples were prepared from the patient and healthy volunteers.

Surface marker analysis. Percentages of lymphocytes bearing Leu-7 or Leu-11 were counted by flow cytometry using anti-Leu-7 and anti-Leu-11 monoclonal antibodies (Becton Dickinson, Sunnyvale, CA).

^{51}Cr release assay. NK cell activity was assayed on ^{51}Cr-labeled K562 cells as previously described.[12,13] In some experiments, normal lymphocytes were incubated with the patient's serum or normal serum at 37°C for 30 minutes, suspended in culture medium with 20% of the serum, and used as effector cells. Cell mixtures, prepared at various effector:target ratios, were brought to a 1-mL final volume and incubated at 37°C for 4, 8, 12, 16, and 24 hours in a CO_2 atmosphere. The following equation was used to express cytotoxicity:percent lysis = [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)] × 100. All assays were set up in triplicate.

Antibody-dependent cell-mediated cytotoxicity (ADCC) was similarly assayed by using a target of Raji cells coated with rabbit anti-Raji antibody.

Single cell–in-agarose assay and morphological examination of target-binding cells. This assay was done according to a modification of the method of Grimm and Bonavida[4,5] as previously described.[12] Effector lymphocytes (2 × 10^5 cells/mL) and unlabeled target K562 (2 × 10^5 cells/mL) were mixed, centrifuged at 500 g for two minutes, and incubated at 37°C for ten minutes. The cell mixture was added to 0.5 mL of 0.5% agarose in a liquid form. The cells and agarose were mixed, and 0.1 mL to 0.2 mL of the mixture was quickly poured onto culture plates that were precoated with 0.5% agarose. After the cell mixture in agarose had solidified, the culture medium was layered and incubated at 37°C for 4, 12, and 18 hours. After the incubation, the medium was removed, and 0.1% trypan blue was added. Percentages of target-binding cells (TBCs) and TBCs with dead targets were determined under a microscope.

Part of the mixture of effector and K562 cells was processed for morphological examination of TBCs. The samples were spun in a cytocentrifuge (Cytospin, Shundon Southern, Elliott, IL) and stained with May-Grünwald-Giemsa staining.

Preparation of cell-free supernatant containing NKCF. NKCF in a cell-free supernatant were prepared by the methods of Wright and Bonavida[6] and Degliantoni et al.[7] The patient's and normal lymphocytes (5 × 10^6 cell/mL) were cultured at 37°C for 18 hours in the absence or presence of 1,000 U/mL recombinant IFN-α (Kyowa Hakko Co) or 1,000 U/mL recombinant IL 2 (Takeda Seiyaku Co). After the culture, the cells were washed and suspended at a concentration of 3 × 10^6 cells/mL in culture medium containing K562 cells (6 × 10^5 final concentration). The cell mixtures were cultured at 37°C for 4, 8, 16, and 24 hours. The cells were then centrifuged, and cell-free supernatants were harvested and processed for cytotoxicity assays.

Assay for NKCF. NKCF in the supernatants were measured by an 18-hour ^{51}Cr-release assay[7] using K562 cells as a target. Briefly, a 50% concentration of the supernatant was added to ^{51}Cr-labeled K562 (5 × 10^3 cells/mL) in a 100-μL final volume in triplicate wells of microtiter plates. After an 18-hour incubation, 100 μL of phosphate-buffered saline was added to each well, and 100 μL of the supernatant was collected after spinning. Cytotoxicity was expressed by the percentage of lysis as described.

RESULTS

Numbers of lymphocytes bearing Leu-7 or Leu-11. The numbers of Leu-7$^+$ cells and Leu-11$^+$ cells were counted in the patient after termination of MOPP therapy. Percentages (mean ± SD) of Leu-7$^+$ cells and Leu-11$^+$ cells were 14% ± 2% and 10% ± 2%, which were normal compared with the normal lymphocyte values of 18% ± 8% and 15% ± 6%, respectively.

Cell-mediated cytotoxicity as determined by ^{51}Cr-release assay. NK cell activity of the patient's lymphocytes was assayed once at the time of diagnosis, once during MOPP therapy, and on seven separate occasions during a 4-year period after termination of the therapy. In every assay, the patient's lymphocytes did not have detectable levels of NK cell activity against NK-sensitive K562 cells: the percent lysis was persistently below 0.3% at a 40:1 effector:target ratio, which was below 0.6% of the normal lymphocyte value of 46.2% ± 5.8% (mean ± SD). Representative results of NK cell activity assays performed in February 1986 are shown in Table 1.

For comparison, NK cell activity was assayed in six children with Hodgkin's disease at the time of diagnosis and/or during a 3-year period after termination of MOPP therapy or radiotherapy. The control patients' lymphocytes at diagnosis had detectable levels of NK cell activity, although the activity was depressed in two of the four patients studied: the percent lysis values in the two were 15.7% and 28.6%, which were below 2 SD of the normal lymphocyte value. The lymphocytes from six patients after termination of the therapy had normal levels of NK cell activity: the percent lysis ranged from 40.1% to 49.2% as compared with the normal lymphocyte value of 46.2% ± 5.8% (mean ±SD).

The patient's lymphocytes did not show NK cell activity at effector:target ratios of 10:1, 20:1, 40:1, and 80:1: the

Table 1. NK Cell Activity Against K562

Effector Cells	Effector Cells Were Stimulated With	cpm*	Percent Lysis
Patient's lymphocytes†	None	571 ± 5	0
	Poly I:C	590 ± 12	0.2
	IFN	623 ± 16	0.7
	IL 2	629 ± 21	0.7
Normal lymphocytes	None	4,313 ± 110	45.9
	Poly I:C	5,951 ± 35	66.0
	IFN	6,115 ± 350	68.1
	IL 2	5,600 ± 442	61.7

*cpm spontaneous release, 570; cpm maximal release, 8,717.

†The cells were obtained 40 months after termination of MOPP therapy.

percent lysis values were below 0.5% at all of the effector:target ratios.

Next, experiments were performed to examine whether the patient's NK cells could respond to in vitro poly I:C, IFN, or IL 2. The results are shown in Table 1. Stimulation of the patient's lymphocytes with poly I:C, IFN, or IL 2 did not significantly augment their cytotoxicity.

The kinetics of NK cell activity of the patient's lymphocytes were tested for cytolysis against K562 target cells at a 40:1 effector:target ratio at five time points over a 24-hour period. As depicted in Fig 1, the patient's lymphocytes did not show cytotoxicity at any time point of the assay. This result certainly precluded the possibility of their delayed killing as a cause of the lack of NK cell activity in the conventional four-hour assay. The responsiveness of the patient's lymphocytes to IFN was also studied under an identical condition. Stimulation of the patient's lymphocytes with IFN did not increase their cytotoxicity at any time point (Fig 1).

Experiments were performed to determine whether the patient's serum had inhibitory effects on normal NK cell activity. There was no difference in cytotoxicity between normal lymphocytes treated with the patient's serum and those treated with normal serum: the percent lysis by the former cells was 96.2% and that by the latter cells was 97.0% of the control value obtained without the serum treatment (Table 2). This result indicated that the lack of NK cell activity was not due to a serum inhibitory factor(s) but a cellular defect.

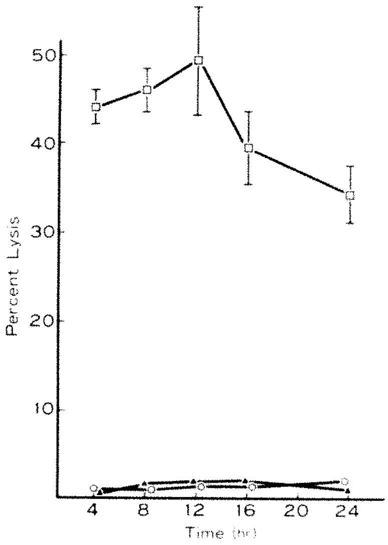

Fig 1. Kinetics of NK cell activity. The patient's lymphocytes (O—O) and normal lymphocytes (□—□) were mixed with ^{51}Cr-labeled K562 target cells at 40:1, incubated for 4, 8, 12, 16, and 24 hours, and assayed for their cytotoxicity. A portion of the patient's lymphocytes (▲—▲) at a concentration of 3 × 10^6/mL was incubated with 1,000 U/mL recombinant IFN-α at 37°C for 20 hours and processed for the cytotoxicity assay. The patient's lymphocytes did not show apparent cytotoxicity at any time point of the assay. Stimulation of the patient's lymphocytes with IFN did not improve the activity. These experiments were performed after termination of MOPP therapy.

Table 2. Effect of Serum on NK Cell Activity Against K562

Effector Cells	Effector Cells Were Treated With	cpm*	Percent Lysis
Normal lymphocytes	None	4,615 ± 120	47.0
	Patient's serum†	4,464 ± 98	45.2
	Normal serum	4,500 ± 131	45.6

*cpm spontaneous release, 620; cpm maximal release; 9,120.

†The serum was obtained after termination of MOPP therapy.

ADCC of the patient's lymphocytes was assayed against Raji cells that were coated with anti-Raji antibody when the patient was not receiving therapy. The patient's lymphocytes had normal levels of cytotoxicity against the target: the percent lysis (mean ± SD) was 21.4% ± 2.6% as compared with the normal lymphocyte value of 26.3% ± 3.6% at a 20:1 effector:target ratio.

NK cell activity as determined by single cell-in-agarose assay and morphology of TBCs. Because NK cells kill target cells after binding to them, experiments were performed after termination of MOPP therapy to determine whether the patient's lymphocytes contained NK cells capable of binding to K562 (TBCs) and, if present, whether they could kill them (TBCs with dead targets) at the single-cell level. In the patient's lymphocytes, the percentage of TBCs was normal: the value (mean ± SD) was 9.3% ± 2.0% as compared with the normal lymphocyte value of 8.3% ± 1.3%. There were no TBCs with dead targets, however, after incubation for 4, 12, and 18 hours in the patient's lymphocytes: the value was below 1% of TBCs as compared with the normal lymphocyte value of 25.6% ± 3.2% (mean ± SD) after a four-hour incubation.

Since NK cells are morphologically identifiable as large granular lymphocytes,[14] morphological observations were performed to examine whether TBCs had the morphology of NK cells. Most of the patient's TBCs as well as normal TBCs contained azurophilic granules in the abundant cytoplasm. Thus these results provided a line of evidence for an NK cell origin of the patient's TBCs that were present in a normal number and were capable of recognizing and binding to K562 cells but were incapable of killing them.

NKCF release. NKCF activity was measured by an 18-hour ^{51}Cr-release assay on K562 cells. The results are shown in Fig 2. The patient's lymphocytes did not release detectable levels of NKCF activity at several time points of the culture over a 24-hour period, which indicated a deficiency of NKCF production by the patient's NK cells. The defect could not be improved by lymphocyte treatment with IFN or IL 2 in vitro.

DISCUSSION

Our patient is characteristic in persistently lacking NK cell activity in every phase of Hodgkin's disease, including the remission phase and while not receiving therapy. There was no restoration or improvement of the NK cell defect by lymphocyte treatment with poly I:C, IFN, or IL 2 in vitro. In this patient, we have demonstrated a killing defect of NK cells with the absence of NKCF.

When NK cells work as effector cells for cytolysis, they first recognize and bind to a target cell,[4,5] and then the NK cells kill the bound target by releasing lytic materials such as

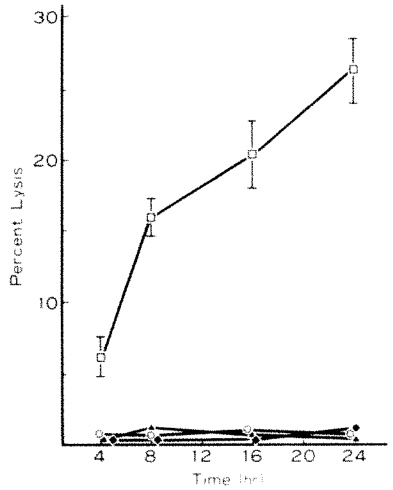

Fig 2. NKCF activity as measured by an 18-hour ^{51}Cr-release assay on K562 cells. The patient's lymphocytes, at a concentration of 5 × 10^6 cells/mL, were cultured at 37°C for 18 hours in the absence (O—O) or presence of 1,000 U/mL recombinant IFN-α (▲—▲) or 1,000 U/mL recombinant IL 2 (♦—♦). Normal lymphocytes (□—□) were cultured in an identical fashion without IFN or IL 2. After the culture, the cells were suspended at a concentration of 3 × 10^6 cells/mL in culture medium containing K562 cells (6 × 10^5 cells/mL final concentration) and cultured for 4, 8, 16, and 24 hours. The cell mixtures were then centrifuged, and cell-free supernatants were obtained. The supernatants were immediately assayed for NKCF activity by an 18-hour ^{51}Cr-release assay using K562 cells as a target. The patient's lymphocytes did not release detectable levels of NKCF at any time point of culture. IFN and IL 2 did not have augmentation effects on the NKCF release by the patient's lymphocytes. These experiments were performed after termination of MOPP therapy.

NKCF[6,7] and NK granule cytolysin[8,9] that selectively lyse NK-sensitive target cells. Our patient had normal numbers of peripheral lymphocytes bearing Leu-7 or Leu-11 antigen, which is closely associated with circulating NK cells.[15,16] In addition, the number of TBCs was normal, and as NK cells, they had azurophilic granules in the cytoplasm.[14] These results thus indicated that the patient had normal numbers of NK cells capable of recognizing a target cell and binding to the cell. Based on the evidence, we thought that a killing step by the NK cells might be defective; this was indeed the case. At the single-cell level, there were no TBCs with dead targets, thereby confirming the lack of their killing capacity. Because NKCF is known to be involved in the killing of a bound target cell by NK cells,[6,7] we next examined the capacity of the patient's NK cells to release NKCF after contact with NK-sensitive K562 cells. The patient's NK cells totally lacked the NKCF release, and the NK cell defect could not be restored or improved by their stimulation with IFN or IL 2. It is suggested that the deficient release of NKCF in this case may have been related to the killing defect of the NK cells. Whether the deficient release of NKCF is due to the absence of the factors within the cells or due to defective secretion of the factors remains to be studied further. However, the normal production of IL 1 and IL 2 by the patient's monocytes and lymphocytes (data not shown) can preclude a broad secretory defect in the patient's cells and may favor the former possibility.

NK granule cytolysin has a potent lytic activity against NK-sensitive target cells, and its activity is functionally different from that of NKCF in potency, target cell selectivity, kinetics of cytolysis, and calcium requirement.[8,9] It would be interesting, therefore, to study NK granule cytolysin in our patient. Unfortunately, we could not study it in this patient because it was difficult to obtain sufficient numbers of NK cells (large granular lymphocytes). The total absence of NK cell activity indicates some abnormality of NK granule cytolysin in the patient's NK cells, although their cytoplasmic granules were morphologically normal, and further studies are expected to confirm this.

Our patient may have acquired the NK cell deficiency as one of the cell-mediated immune defects in Hodgkin's disease,[17] but why he has such a persistent and profound immunodeficiency remains obscure. In some patients with Hodgkin's disease, NK cell activity is depressed before and/or during chemotherapy or radiotherapy[18-21] but becomes normal after attaining complete remission or after termination of therapy.[19,21] Actually, some of our control patients with Hodgkin's disease had depressed but detectable levels of NK cell activity at diagnosis, and all of them had normal levels of NK cell activity after therapy. It is known that depression of NK cell activity is not related to the clinical stage or histological type of Hodgkin's disease[20]; this was the same in our studies. Serum inhibitory factors are often described in this disease,[22] but the possibility of such factors as a cause of the NK cell deficiency can be excluded by the absence of inhibitory effects of the patient's serum on the normal NK cell function, as demonstrated here. This result further indicates that the NK cell deficiency is a cellular defect in our patient.

Alternatively, the patient may have an intrinsic cellular defect of NK cells. His history of recurrent infections since infancy appears to favor this possibility. With respect to this, the patient's parents and brother had normal levels of NK cell activity (data not shown), which provided no definite evidence for familial occurrence of the NK cell deficiency.

It is noteworthy that ADCC was normal despite the absence of NK cell activity in our patient. Such a discrepancy between NK cell activity and ADCC has also been demonstrated in Hodgkin's disease.[18] These findings certainly support the evidence that NK cells are distinct from K cells in ADCC,[23] although they have similar cellular characteristics.[14,24]

The clinical significance of the NK cell deficiency in our patient is of interest because NK cells provide an important defense against viral infections and tumor development.[2,3] In fact, the patient has suffered from recurrent infections, which may be largely due to the NK cell deficiency. Further studies will determine whether such NK cell deficiency is related to the high incidence of second malignant neoplasms in Hodgkin's disease.

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The Effect of ABO Blood Group on the Diagnosis of von Willebrand Disease

By Joan Cox Gill, Janet Endres-Brooks, Patricia J. Bauer, William J. Marks, Jr, and Robert R. Montgomery

In order to firmly establish a normal range for von Willebrand factor antigen (vWF:Ag), we determined plasma vWF:Ag concentrations in 1,117 volunteer blood donors by quantitative immunoelectrophoresis. The presence of the ABO blood group had a significant influence on vWF:Ag values; individuals with blood group O had the lowest mean vWF:Ag level (74.8 U/dL), followed by group A (105.9 U/dL), then group B (116.9 U/dL), and finally group AB (123.3 U/dL). Multiple regression analysis revealed that age significantly correlated with vWF:Ag levels in each blood group. We then performed reverse ABO typing on stored plasma from 142 patients with the diagnosis of von Willebrand disease (vWd). Of 114 patients with type I vWd, blood group O was found in 88 (77%), group A in 21 (18%), group B in 5 (4%), and group AB in none (0%), whereas the frequency of these blood groups in the normal population is significantly different (45%, 45%, 7% and 3%, respectively) ($P < .001$). Patients with type II or III vWd had ABO blood group frequencies that were not different from the expected distribution. There may be a subset of symptomatic vWd patients with decreased concentrations of structurally normal vWf (vWd, type I) on the basis of blood group O. Some individuals of blood group AB with a genetic defect of vWF may have the diagnosis overlooked because vWF levels are elevated due to blood type.

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THE DIAGNOSIS of type I von Willebrand disease (vWd) is often based on the demonstration of a decreased quantity of structurally intact plasma von Willebrand factor (vWF) in a patient with a history of mild mucocutaneous or surgically induced hemorrhage.[1-3] The diagnosis can be difficult in mildly affected individuals because vWF levels tend to fluctuate, often rising into the normal range during times of stress, inflammation, pregnancy, and ingestion of oral contraceptives.[1-7] Furthermore, firmly established normal ranges based on von Willebrand factor antigen (vWF:Ag) determinations in a large cross section of normal individuals are lacking.

The quantity of vWF:Ag assayed, as for most coagulation factors, is expressed in relation to a standard derived from a pool of plasma from normal donors, with the pool representing 100 U/dL. Assuming a normal distribution, if the number of individuals in a particular pool is large enough, then high normals should be offset by low normals, and the concentration of a particular plasma constituent should approach the mean. Traditionally, the mean is defined as 100 U/dL, and a vWF:Ag value below 40 to 50 U/dL is used as a criterion for the diagnosis of vWd.

In preliminary studies in our laboratory, five of 100 standard pool members were found to have plasma vWF:Ag values less than 50 U/dL. This finding prompted us to study a much larger group of normal individuals. To firmly establish a normal range for vWF:Ag and to further investigate factors that might influence plasma vWF levels, we studied vWF:Ag in 1,117 blood donors.

MATERIALS AND METHODS

Collection of blood samples. Plasma was obtained from venous blood at the time of volunteer blood donation. After the unit of whole blood had been collected in the usual manner, the plastic tubing between the needle and the bag was sealed. The tubing was then cut between the seal and the needle and venous blood collected into polystyrene tubes prefilled with sodium citrate (3.2%) so as to result in a 9:1 ratio of whole blood to anticoagulant. Plasma was obtained by centrifugation at 4,800 g for 20 minutes in a refrigerated centrifuge. Samples were stored at −80°C until assayed.

vWF:Ag. Measurement of vWF (vWF:Ag) was performed by using quantitative immunoelectrophoresis in 0.9% agarose and rabbit antihuman vWF as previously described.[8] The reference standard for vWF:Ag was pooled plasma from 50 normal donors (stored at −80°C). All reference standards derived from pooled plasmas were assayed against the World Health Organization standard.

Determination of vWd type. Patients referred for evaluation of clinically significant bleeding had the following laboratory studies performed to determine vWd type: vWF:Ag as described earlier, vWF activity by a ristocetin cofactor assay utilizing formalin-fixed human platelets,[9] factor VIII (F VIII) procoagulant activity by a one-stage method utilizing F VIII–deficient plasma as substrate,[10] and multimeric analysis of vWF by electrophoresis of plasma in 0.65% Seakem high gelling temperature ultra pure (HGT-(P)) agarose gels (FMC Corp, Rockland, ME) in the presence of 0.1% sodium dodecyl sulfate (SDS) and visualized with radiolabeled polyclonal rabbit antihuman vWF and autoradiography as previously described.[11] The diagnosis of type I vWd was based on the finding of proportionally decreased plasma vWF:Ag and ristocetin cofactor activity below 45 U/dL and a normal multimeric pattern of vWF:Ag. Type II vWd was diagnosed by the findings of ristocetin cofactor activity below 45 U/dL, vWF:Ag disproportionately higher than ristocetin cofactor activity, and an abnormal multimeric pattern of vWF characterized by an absence of the high-molecular weight multimers. Patients with undetectable vWF were classified as having type III vWd.

Reverse ABO blood group typing. Plasma from patients with the diagnosis of vWd, stored at −80°C, was mixed with saline-washed RBC from individuals of blood groups A and B, centrifuged, and observed for hemagglutination.

Statistical analysis. Multiple-regression analysis (FPSS Statistical Package for Social Services), one-way analysis of variance, Student's t test, and the goodness-of-fit test were performed as appropriate.

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Table 1. Influence of ABO Blood Group on vWF:Ag Values in Volunteer Blood Donors

ABO Type	n	vWF:AG Geometric Mean	vWF:Ag Geometric Mean ± 2 SD
O	456	74.8	35.6-157.0
A	340	105.9	48.0-233.9
B	196	116.9	56.8-241.0
AB	109	123.3	63.8-238.2

The groups were statistically significantly different from each other as follows: O *v* A, B, and AB, $P < .01$; A *v* AB, $P < .01$; B *v* A, $P < .05$.

RESULTS

vWF:Ag in 1,101 blood donors. Initially, 1,000 sequential blood donors from the Blood Center of Southeastern Wisconsin had determinations of vWF:Ag levels. To increase the number of individuals of ABO groups B and AB, additional sequential samples from 52 group B and 65 group AB donors were collected for a total of 1,117 samples. Twelve donors were excluded because of vasovagal reactions during the donation and four because their vWF:Ag values were beyond 3 SD above the mean (statistical outlyers) for their ABO group after adjusting for age. Statistical analyses were performed with values from the remaining 1,101 donors.

vWF:Ag values were significantly affected by the ABO blood group of donors. As indicated in Table 1, vWF:Ag values in each group were significantly different from the others; group AB individuals had the highest geometric mean vWF:Ag level (123.3 U/dL), followed by group B (116.9 U/dL), then group A (105.9 U/dL), and finally group O individuals with the lowest values (74.8 U/dL).

Multiple-regression analysis of variables other than ABO group including age, gender, Rh type, and the ingestion of medications revealed that in the group as a whole and within each ABO blood group age was the only variable that was significantly predictive of vWf:Ag (Fig 1). Age did not account for the variation in vWF levels among ABO blood groups, since the range and mean age were similar in the groups (Table 2). However, as higher ages were attained, mean vWF:Ag values in persons of blood group A and blood group B approached each other (Fig 2).

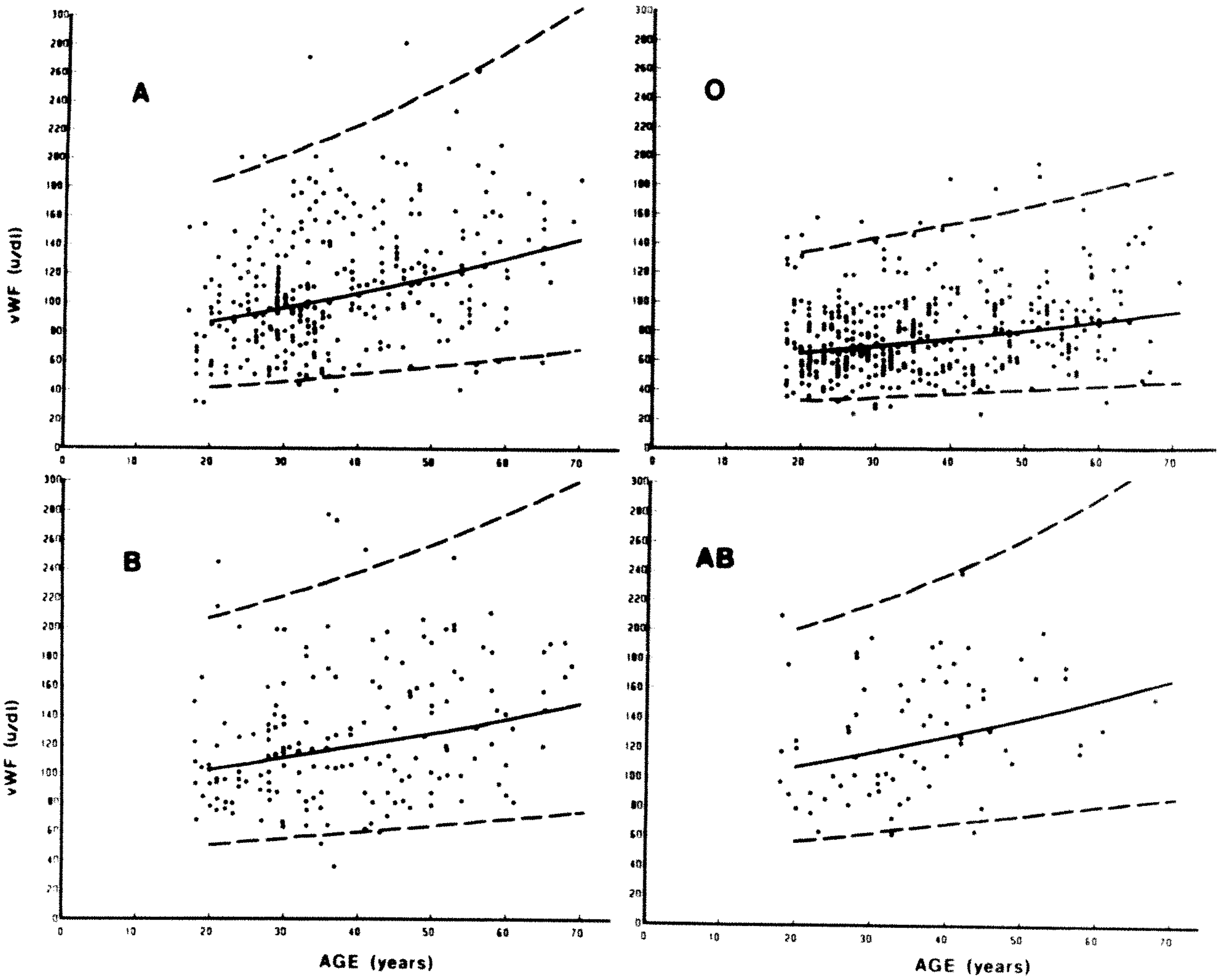

Fig 1. Increase in vWF:Ag with age in volunteer blood donors. ABO blood group is indicated by the letters O, A, B, or AB. Mean vWF *v* age is shown by the solid lines, and 95% confidence intervals are indicated by the broken lines.

Table 2. Range and Mean Age of Blood Donors

Blood Group	Mean Age	Age Range
O	38.2	18-71
A	38.2	17-77
B	38.7	18-69
AB	37.5	18-69

The difference in ages was not significantly different by one-way analysis of variance.

In group A individuals only, when age was accounted for, the male gender ($P < .02$) and ingestion of medications ($P < .05$) were associated with a higher vWF:Ag concentration. Of the 43 blood group A individuals taking medications, the largest group (n = 18) was using antihypertensive drugs, followed by antibiotics (8), oral contraceptives (6), recent immunizations or desensitization with allergens (5), antihistamines (4), and antidepressants (1).

Since the blood sample for vWF:Ag determination was obtained after the regular blood donation, it was possible that the stress associated with the procedure might bias the results. Therefore, 25 donors agreed to undergo a separate venipuncture for vWF:Ag determination immediately prior to the regular donation, with the second sample collected after the blood donation as done in the other donors. The mean predonation vWF:Ag concentration in these donors was 2.5 U/dL higher than the postdonation samples, not significantly different ($P = .80$).

To determine whether structurally abnormal vWF might be present in group O donors with low vWF levels, an additional 104 group O donors were evaluated. Ristocetin cofactor assays correlated well with vWF:Ag with a correlation coefficient of 0.80 ($P < .01$). Multimeric analysis of vWF on SDS agarose gels were performed on the 11 donors from this group of 104 blood group O individuals with a vWF:Ag level <45 U/dL and on three others from this group who had a discrepancy between the ristocetin cofactor and vWF:Ag assays; all 14 patterns were normal.

ABO blood groups in patients with a diagnosis of vWd. Reverse ABO typing of 142 plasma samples from patients carrying a diagnosis of vWd was performed (Table 3). The frequency distribution of ABO blood groups in type I vWd (quantitative deficiency of structurally normal vWf) was significantly different from the expected distribution (personal communication, J.E. Menitove, Blood Center of Southeastern Wisconsin) when analyzed by the goodness-of-fit test ($P < .001$). Of 114 patients with a diagnosis of type I vWd, 88 (77%) had blood group O, whereas the expected frequency of blood group O in the normal population was 45%. In contrast, blood groups A, B, and AB were underrepresented in type I vWd patients when compared with the expected pattern of distribution. When the ABO frequency distribution of type I vWd patients was adjusted by excluding the 23 individuals with vWF values greater than 37, of the 91 total, 65 (71%) had group O, 21 (23%) had group A, 5 (5%) had group B, and none had group AB. This distribution was also significantly ($P < .001$) different from the expected. The 22 patients with a diagnosis of type II vWd (structurally abnormal vWf) had an expected frequency of ABO groups, and the six with type III vWd (undetectable vWF) were too few for a meaningful analysis.

Table 3. Frequency of ABO Blood Groups in Patients With vWd

Patients		Frequency of Each ABO Blood Group			
		O	A	B	AB
Type	n	n (%)	n (%)	n (%)	n (%)
vWd I	114	88 (77)	21 (18)	5 (4)	0 (0)
vWd II	22	7 (32)	10 (45)	3 (14)	2 (9)
vWd III	6	4 (67)	2 (33)	0 (0)	0 (0)
Expected frequency		(45)	(45)	(7)	(3)

The observed ABO frequency pattern in type I vWd was statistically significantly different from the expected frequency ($P < .001$). Type II was not significantly different, and type III patients were too few for a meaningful analysis.

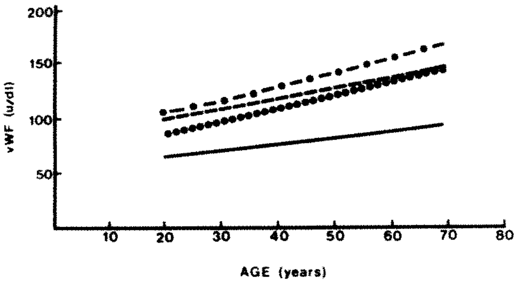

Fig 2. Mean vWF:Ag *v* age in volunteer blood donors. Mean vWF in blood group AB, ●—●; group B, ----; group A, · · · ·; and group O, ——.

DISCUSSION

This study confirms previous reports[12-15] that plasma vWF:Ag concentration is significantly affected by the ABO blood group. As in this study, all but one investigator found that blood group O was associated with the lowest vWF:Ag values; the single exception was a small study conducted by Mohanty and coworkers,[14] who found the lowest vWF:Ag values in blood group B individuals and suggested that this difference was due to ethnic variation. In the studies by McCallum and coworkers[13] of female blood donors and Stormorken and Erikssen[12] of middle-aged men, no significant differences in vWF:Ag values were found between individuals of blood groups A, B, or AB, whereas this study demonstrated that the vWF:Ag concentration in group AB was highest, lower in group B, and lower still in group A. The smaller sample size (136) of McCallum and colleagues' study and the restriction of the sample to middle-aged men in Stormorken and Erikssen's study may account for these differences.

In a study of monozygotic and dizygotic twins, Orstavik and coworkers[15] determined that F VIII:C was dependent on vWF:Ag levels and that 30% of the genetic variance of vWF:Ag was due to the effect of ABO blood group. The twins were all greater than 33 years of age, and those with blood group A_1 had higher vWF:Ag levels than those with group B in whom levels were higher than group A_2. These findings differ from our investigation but could be explained by the differences in age in the two samples and our finding that vWF:Ag values in group A and group B individuals approach each other as age increases; furthermore, we did not differentiate between groups A_1 and A_2.

Genetic linkage analyses previously suggested that the loci for vWF and ABO blood groups are not linked.[16,17] This conclusion has been substantiated by the recent location of the gene for vWF on chromosome 12,[18] whereas the gene for ABO blood group determination is on chromosome 9.[19-21] Thus, a direct linkage could not explain the significant variation in plasma vWF concentration among individuals of different ABO blood groups.

vWF is a highly glycosylated protein[22,23]; its platelet-agglutinating activity in the presence of ristocetin,[24-29] susceptibility to proteolytic degradation,[30] and survival in the normal rabbit circulation[31] have been shown to be affected by the removal of sialic acid or galactose residues, which suggests that carbohydrate is important in the structure/function of vWF. Furthermore, blood group A, B, and H oligosaccharide structures have been identified on human vWF.[23] It is possible that the blood group determinants may affect the processing, release, or catabolism of vWF, thus influencing the plasma concentration of the protein.

Since ABO blood group was found to be a significant determinant of plasma vWF concentration, we wondered whether the diagnosis of vWd was influenced by the patient's ABO status and found that significantly more type I vWd patients than expected had blood group O. The additional finding of increased numbers of group O individuals when the diagnosis was based on ABO-adjusted normal ranges suggests that there may be a subset of type I vWd patients with decreased concentrations of structurally normal vWF on the basis of blood group rather than specific inherited abnormalities of vWF production or release. Similarly, individuals of blood group AB with a vWF genetic defect may have the diagnosis overlooked because the vWF is elevated due to blood type.

We do not intend to imply that symptomatic individuals with vWF in the range of 37 to 45 U/dL and blood group O do not have a form of vWd and should not be treated as such. The treatment of such individuals, as in any clinical situation, should not be altered because of this theoretical consideration. However, the inheritance of vWd in such an individual may be different than in an individual with a specific vWF gene abnormality. Thus, the mechanism of decreased concentrations of plasma vWF in various patients may be heterogeneous; further clarification of this issue may evolve as specific chromosomal abnormalities resulting in type I vWd are defined.

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Type I Glanzmann Thrombasthenia Patients From the Iraqi-Jewish and Arab Populations in Israel Can Be Differentiated by Platelet Glycoprotein IIIa Immunoblot Analysis

By Barry S. Coller, Uri Seligsohn, and Pamela A. Little

A sensitive immunoblot technique for platelet glycoprotein IIIa (GPIIIa) was used to analyze the platelets of patients living in Israel who meet the diagnostic criteria for type I Glanzmann thrombasthenia. When reacted with solubilized normal platelets, a rabbit antiserum to GPIIIa identified a major band at molecular weight (mol wt) 90,000 and three additional minor bands at Mr 110,000, 81,000, and 64,000. The major band could not be detected, and the minor bands were either markedly reduced or absent in the platelet samples from 14 of the 15 patients from the Iraqi-Jewish population. In contrast, in all four Arab patients tested, the major band was detectable, although at markedly reduced levels, and the minor bands were either markedly reduced or absent; an additional minor band at mol wt 47,000 was also present in the platelets from these patients. One Iraqi-Jewish patient had a unique pattern in which two of the bands were present but reduced and two were undetectable. We conclude that the protein defect, and thus presumably the genetic defect, causing Glanzmann thrombasthenia in the majority of patients in the Iraqi-Jewish population differs from that in the Arab population, and we confirm that there is considerable biochemical heterogeneity among the patients who meet the criteria for type I Glanzmann thrombasthenia.

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GLANZMANN thrombasthenia is an autosomal recessive disorder of platelet function, characterized by excessive hemorrhage from mucocutaneous sites.[1-3] Laboratory features include decreased or absent platelet functions, including clot retraction; adhesion to glass; and platelet aggregation in response to ADP, epinephrine, and thrombin.[1-3] Affected platelets also fail to interact normally with both fibrinogen and von Willebrand factor (vWF) when stimulated with ADP or thrombin.[4-8] The discovery of a deficiency of the glycoprotein IIb/IIIa complex in thrombasthenic platelets[3,9,10] provided a molecular basis for the disease, since there is evidence that this complex can function as a receptor for fibrinogen,[3,11] vWF,[7,8] and fibronectin.[12] Patients who meet the minimal clinical and laboratory criteria for Glanzmann thrombasthenia (a lifelong bleeding diathesis associated with absent platelet aggregation in response to adenosine diphosphate [ADP] and decreased clot retraction), have, however, displayed considerable heterogeneity in clinical symptoms and laboratory data,[1,13,14] leading to attempts to categorize the disease into different types.[15] Patients with type I Glanzmann thrombasthenia are said to have absent clot retraction and little or no platelet fibrinogen, whereas patients with type II are said to have some clot retraction and readily detectable, if somewhat reduced, platelet fibrinogen.[15] By crossed immunoelectrophoresis and electroimmunoassay, several patients with the laboratory criteria for type I thrombasthenia had no detectable GPIIb/IIIa complex, whereas several patients who met the criteria for type II had reduced but detectable levels of the complex.[16-18]

Early studies of the platelet glycoproteins in Glanzmann thrombasthenia patients utilizing polyacrylamide gel electrophoresis to separate the glycoproteins, which were either prelabeled before solubilization or detected with stains specific for carbohydrate and/or protein after electrophoresis, suggested that most patients shared the same abnormality, namely, a marked reduction but probably not absence of both GPIIb and GPIIIa.[10,19,20] Subsequent studies indicated that there was more heterogeneity in glycoprotein patterns, with some patients having no detectable GPIIb and GPIIIa, others having discordant amounts of the two glycoproteins, and others having considerable amounts of both glycoproteins.[21-26] Most recently, Nurden studied seven type I and two type II thrombasthenic patients with the technique of electroblotting of proteins separated by polyacrylamide gel electrophoresis onto nitrocellulose paper followed by immunologic detection of the glycoproteins with specific heterologous antisera.[27] As expected, the type II patients had readily detectable GPIIb and GPIIIa. The type I patients, however, had unexpected heterogeneity, with only one demonstrating the absence of both GPIIb and GPIIIa. These data emphasized the rarity of the complete absence of both glycoproteins, even in type I patients, and highlighted the insensitivity of the crossed immunoelectrophoresis technique for quantifying the individual glycoproteins.

We recently reported the immunologic and biochemical characterization of the platelets of 32 patients from 19 families with thrombasthenia from the Iraqi-Jewish and Arab populations in Israel.[28] All 19 patients whose platelets were analyzed by polyacrylamide gel electrophoresis were devoid of GPIIb, as judged by carbohydrate and protein staining. Patient platelets bound only ~2% of the normal amount of a monoclonal antibody (MoAb; 10E5) directed against GPIIb and/or GPIIIa; this antibody reacts with the glycoproteins when they are complexed with another but not

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when the complex is dissociated with calcium chelating agents.[29,30] In fact, it was unclear whether this minimal amount of antibody binding represented a significant increase above background. GPIIIa could not be assessed in that study because platelet samples for polyacrylamide gel electrophoresis were analyzed in the nonreduced state, and under this condition another glycoprotein (GPIIb) comigrates with GPIIIa. In the present study we have analyzed 15 patients from 11 families in the Iraqi-Jewish population and four patients from four families in the Arab population with an immunoblot technique using a heterologous antibody to GPIIIa and a highly sensitive immunogold/silver enhancement detection technique. Our studies demonstrate one major immunoreactive band that appears to be related to GPIIIa and three minor bands that may be related to GPIIIa. In all 15 Iraqi-Jewish patients, the major GPIIIa band was not detectable. In 14 of the 15 patients the minor bands were either reduced or absent, whereas in one patient two of the minor bands were more prominent. In sharp contrast, all four Arab patients had reduced but detectable levels of the major GPIIIa band, reduced or absent minor bands, and an additional band of lower molecular weight (mol wt). We conclude that different protein and thus presumably genetic abnormalities produce the thrombasthenic syndrome in the Iraqi-Jewish and Arab populations in Israel even though the patients in both groups meet the traditional criteria for type I disease.

MATERIALS AND METHODS

Patients. The patients investigated in this study come from the populations previously described.[28,31] In all, 15 Iraqi-Jewish patients from 11 families and four Arab patients, each from a different family, were studied.

Platelet preparation. Platelets from citrated blood were washed as previously described,[28] resuspended to ~4 × $10^6/\mu L$, and solubilized with an equal volume of 3.3% sodium dodecyl sulfate (SDS)-6 mmol/L N-ethylmaleimide, as previously described.[28] Samples were stored frozen at −20°C. Control platelets samples were obtained from normal individuals both in Israel and at Stony Brook; the former were prepared earlier, at the same time as the patient samples, whereas the latter were prepared later, within weeks of when the samples were assayed by the immunoblot technique. No differences were observed between these different controls.

Antibodies

Rabbit anti-GPIIIa serum. GPIIIa was purified from human platelets by a modification of the differential NaCl extraction technique of Jennings and Phillips[32] and was used to immunize a rabbit as follows. Six units of outdated platelet concentrates (kindly provided by Dr Dennis Galanakis of the blood bank at the State University of New York at Stony Brook Hospital) were pooled, made 1 mmol/L in EDTA, and washed two times with 0.15 mol/L NaCl, 0.01 mol/L Tris, 1 mmol/L EDTA, pH 7.4. The platelets were divided in half and pelleted separately. One pellet (A) was resuspended in 25 mL of the above buffer and the other (B) was resuspended in 25 mL of 0.15 mol/L NaCl, 0.01 mol/L Tris, 1 mmol/L $CaCl_2$, pH 7.4. The suspensions were kept on ice, and Triton X-100 was added to bring the final concentration to 0.1%. After 55 minutes, the suspensions were centrifuged at 80,000 g for 60 minutes at 4°C. The pellets were brought up in 10 mL of the above buffers, but in each case the NaCl was omitted. The suspensions were again made 0.1% in Triton X-100 and allowed to incubate on ice for 60 minutes. The samples were then recentrifuged at 80,000 g for 60 minutes at 4°C. The supernatants, which contained approximately the same amounts of both GPIIb and GPIIIa as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), were then chromatographed individually on a 90 × 2.5 cm column of Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) with an elution buffer of 0.05 mol/L sodium phosphate, 0.05% Triton X-100, and 0.05% sodium azide. The fractions from both columns that contained the GPIIa/IIIa complex were pooled, made to contain 10 mol/L EDTA and 0.2% SDS in an attempt to dissociate GPIIb from GPIIIa, and then rechromatographed on the Sephacryl S-300 column with an elution buffer (0.15 mol/L NaCl, 0.01 mol/L Tris, 10 mmol/L EDTA, 0.2% SDS, pH 7.4) designed to maintain the separation of GPIIb from GPIIIa. The fractions containing purified GPIIIa, as judged by the presence of a single band by silver staining after SDS-PAGE, were pooled, and subsamples were removed for digestion by pepsin. First, 0.11 volume of 2 mol/L NaCl, 2 mol/L Na acetate, pH 4.0 was added to the sample (2 mL), and then 22 μL of pepsin A (1 mg/mL in 0.2 mol/L NaCl, 0.2 mol/L Na acetate, pH 4; Worthington Biochemical Co, Freehold, NJ) was added, bringing the final concentration to 10 μg/mL. The digest was kept at 37°C, and additional aliquots of pepsin were added at 1.3 hours, 2.3 hours, 17.6 hours, and 25.3 hours. After digestion, no intact GPIIIa could be detected by SDS-PAGE. A mixture consisting of 1 mL of undigested GPIIIa and 0.75 ml of pepsin-digested GPIIIa was then emulsified with an equal volume of complete Freund's adjuvant (Sigma Chemical, St. Louis). The back of an albino rabbit was shaved, and 0.1 mL of the emulsion was injected intradermally at multiple sites via a 25-g scalp infusion set with the aid of a syringe infusion pump (model 975, Harvard Apparatus, Millis, MA). The rabbit was reinjected on day 11, day 21, and day 71. On day 80, the animal was anesthetized, and approximately 120 mL of blood was obtained via cardiac puncture. The blood was allowed to clot at 37°C for three hours in a glass tube, after which the serum was decanted, centrifuged at 2,000 g for ten minutes at 22°C to remove residual erythrocytes, and the supernatant serum heat-inactivated at 60°C for 20 minutes before being aliquotted and frozen at −20°C.

Murine monoclonal anti-GPIIIa antibody. This antibody, designated 16, was kindly supplied by Dr Simon Karpatkin of New York University School of Medicine. The antigen was partially purified from washed platelets by selective detergent solubilization in Triton X-114[33] followed by final purification by SDS-PAGE. The portion of the gel containing the GPIIIa band was excised and the protein eluted from the gel; it was then used to immunize a mouse. The latter's splenocytes were fused to a mouse myeloma cell line by conventional methods and the supernatants assayed for reactivity with GPIIIa. For the present studies, culture supernatants were precipitated with 50% ammonium sulfate, after which the precipitates were dissolved in one-fourth the original volume of phosphate-buffered saline (PBS) containing 0.025% sodium azide and then dialyzed, first against water and then against the same phosphate buffer, before being frozen.

Human anti-Pl^{A1} antibody. This antibody (also kindly supplied by Dr Simon Karpatkin) was from a male patient who developed thrombocytopenia after cardiac surgery as a result of post-transfusion pupura.[34] The patient's serum was found to contain an antibody (or antibodies) reactive with the Pl^{A1} antigen, which is known to reside on GPIIIa.[35] The patient's plasma was obtained by plasmapheresis and a fourfold concentrated immunoglobulin fraction was prepared as described above for the MoAb.

SDS-PAGE. Solubilized platelets were mixed 2:1 with a sample buffer containing 3.05% SDS, 0.18 mol/L Tris/Cl, 30% glycerol, and 0.02% bromophenol blue, after which they were heated to 100°C for three minutes. A 38-μL sample containing ~150 μg platelet protein was then applied to a 1.5-mm thick gel containing a 1 × 10-cm 3% acrylamide stacking gel and a 5.4 × 10-cm 7% resolving gel containing the discontinuous buffer system of Laemmli.[36] The running buffer was 0.05 mol/L Tris/glycine, 0.4% SDS, pH 8.9. Electrophoresis was carried out at 50 V until the sample entered the separation gel (Mighty Small slab gel electrophoresis unit, SE 200, Hoefer Scientific Instruments, San Francisco) (~30 minutes); the voltage was then increased to 100 to 150 V and electrophoresis proceeded until the blue tracking dye approached the bottom of the gel. Earlier samples were electrophoresed at 150 V, but this was found to result in broadening of the protein bands. When similar gels were stained with a silver reagent (Bio-Rad silver stain kit, Bio-Rad Laboratories, Richmond, CA), which only stains the protein on the outer surfaces of the gel, it was found that the protein on the gel surface closer to the cooling plate migrated less rapidly than the protein on the gel surface exposed to the ambient air. Thus the broadening was presumed to reflect differential thermal effects, and this was minimized by decreasing the voltage to 100 V and heating the running buffer to 37°C. Proteins of known mol wt (Sigma Chemical Co., St. Louis) were also electrophoresed on each gel (carbonic anhydrase 29,000; egg ovalbumin, 45,000; bovine plasma albumin, 66,000; rabbit muscle phosphorylase B, 94,000; *Escherichia coli* β-galactosidase, 116,000; and rabbit muscle myosin, 250,000).

Immunoblotting. After electrophoresis was finished, the lane containing the mol wt standards was cut from the gel and stained with Coomassie blue; the proteins contained within the remainder of the gel were transferred to nitrocellulose paper (0.2 μ pore size; Schleicher and Schuell, Keene, NH) using an electrotransfer apparatus (Mini Transphor; TE22; Hoefer Scientific Instruments) according to a modification of the technique described by Towbin et al.[37] In particular, two pieces of paper were placed anodally to the gel, and one was placed cathodally; only the first piece placed anodally was used for immunoblotting, whereas the others were washed in PBS pH 7.2 containing 0.3% Tween 20 and then stained with a colloidal gold reagent (AuroDye, Janssen Life Sciences Products, Beenge, Belgium) to assess the completeness of the transfer. The transfer buffer (0.192 mol/L glycine, 25 mmol/L Tris, pH 8.3 containing 20% [vol/vol] methanol) was thoroughly degassed and cooled to 4°C before use; electrophoresis was carried out with tap water cooling (~18°C) at 400 m Amps for one hour. Coomassie blue staining of the gel after transfer showed that there was nearly complete loss of proteins below mol wt 180,000 and partial loss of higher mol wt proteins.

The immunoreactive bands on the nitrocellulose paper were then detected by reaction with a gold-conjugated anti-immunoglobulin antibody and subsequent enhancement of the gold stain with a silver reagent (AuroProbe BL IGSS kit; Janssen). The nitrocellulose was incubated with a blocking solution (20 mmol/L Tris/Cl, 0.9% NaCl, 5% bovine serum albumin [BSA], 20 mmol/L sodium azide, pH 8.2) for two hours at 37°C and then overnight at 4°C, after which it was washed with the same Tris-NaCl buffer containing 0.1% BSA. The paper was then incubated with gentle shaking for two hours at 22°C with dilutions of the human anti-Pl^{A1} immunoglobulin fraction (1/1,000), the monoclonal mouse anti-GPIIIa immunoglobulin fraction (1/2,500), or the rabbit anti-GPIIIa serum (1/50) in the Tris-NaCl, 0.1% BSA, pH 8.2 buffer to which 1% normal goat serum was added. After washing in Tris-NaCl, 0.1% BSA buffer three times, the paper was incubated with gentle shaking with 1/100 dilutions of affinity-purified goat antibodies to human, mouse, or rabbit immunoglobulins that were conjugated to 20 mmol/L colloidal gold particles. The antibodies were diluted in Tris-NaCl, pH 8.2 buffer containing 0.4% gelatin (wt/vol) and 20 nm sodium azide and the incubation proceeded overnight at 22°C. Light pink bands could be detected at this point. After washing with the Tris-NaCl, 0.1% BSA buffer, the intensity of the bands was enhanced by reaction with a freshly prepared silver stain (IntenSE, Janssen) that precipitates metalic silver on the gold surface and converts the pink bands to a gray-black color. Equal volumes of the enhancer (4.7 g/100 mL) and initiator (7.8 g/100 ml) solutions were mixed immediately before use and the nitrocellulose paper was stained for approximately ten minutes at 22°C. The paper was then placed in the fixer for five to ten minutes, after which it was washed in water and dried. The silver enhancement step led to some variability in the intensity of individual bands between blots, and so the current experiments were performed such that the control and patient samples were included on each blot, and each blot was developed as a unit.

Radioimmunoelectrophoresis. This technique was conducted as previously described using a combination of the rabbit anti-GPIIIa serum and radiolabeled 10E5.[38,39] Platelet samples solubilized in Triton X-100 were obtained from a normal individual and a patient from the Iraqi-Jewish population.

RESULTS

Characterization of the rabbit antiserum to GPIIIa. The antiserum gave only a single arc against normal platelets when tested in radioimmunoelectrophoresis as judged by Coomassie blue staining. This arc was identified as containing the GPIIb/IIIa complex, since the radioautogram of the same gel showed unique incorporation of radiolabeled 10E5 into this arc, and no arc was present by either detection method when a sample of platelets from a patient with Glanzmann thrombasthenia was tested.

Comparison of rabbit anti-GPIIIa, human anti-Pl^{A1}, and mouse monoclonal anti-GPIIIa antibodies. All three antibody preparations recognized three proteins in nonreduced platelet samples, and these are designated 1, 2, and 3 in Fig 1. Band 2 was much more intense than the other two bands and corresponded in mol wt to the nonreduced value of purified GPIIIa (mol wt ~90,000). Studies using the rabbit

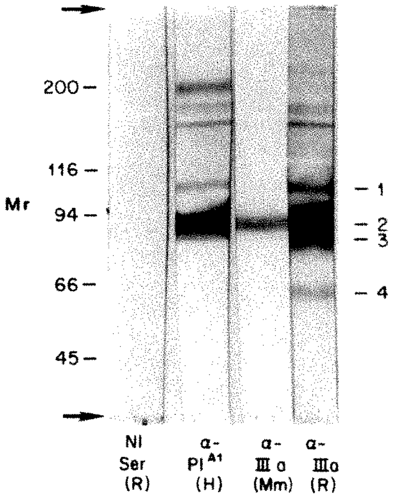

Fig 1. Immunoblot analysis of normal platelet proteins reacted with normal rabbit serum (Nl Ser [R]), a human anti-Pl^{A1} immunoglobulin fraction (α-Pl^{A1} [H]), a mouse MoAb to GPIIIa (α-IIIa [Mm]), and a rabbit anti-GPIIIa antibody (α-IIIa [R]). The mol wt of the reference proteins are indicated on the left (in kilodaltons), and the four immunoreactive bands are indicated on the right.

antiserum and dilutions of the control platelet sample showed that this band could be detected at a dilution of 1/1,000, indicating a sensitivity in excess of 0.1%. Band 1 was of mol wt ~110,000, and band 3, which could not always be resolved from band 2, was of mol wt ~81,000. An additional band of mol wt ~64,000 was recognized by the rabbit anti-GPIIIa serum, but not by the other two immunoglobulin preparations. A variety of higher mol wt bands were also visible with both the rabbit anti-GPIIIa and human anti-Pl^{A1} antibodies. One of these bands (mol wt ~160,000) was visible with the mouse monoclonal anti-GPIIIa antibody and to a variable extent with normal rabbit serum, and so it was believed to represent cross reactivity of the second antibodies with the human IgG present in the platelet preparations. Some of the other high mol wt bands and some bands in the mol wt 80,000 to 90,000 range were also visible to a lesser extent when normal rabbit serum was used instead of the immune serum, and so they were not considered specific. When platelet samples were reduced with 5% β-mercaptoethanol before SDS-PAGE, no bands were detected with any of the three different immunoglobulin preparations. This probably results from a marked change in the tertiary structure of this cysteine-rich protein, which also undergoes a large increase in mol wt with reduction.

Comparison of immunoblot patterns of platelet proteins from Iraqi-Jewish and Arab patients with thrombasthenia. The immunoblots of control and patient platelets reacted with the rabbit anti-GPIIIa serum are shown in Fig 2. The platelet sample from controls 1, 5, and 6 were prepared at Stony Brook, whereas those from controls 2, 3,

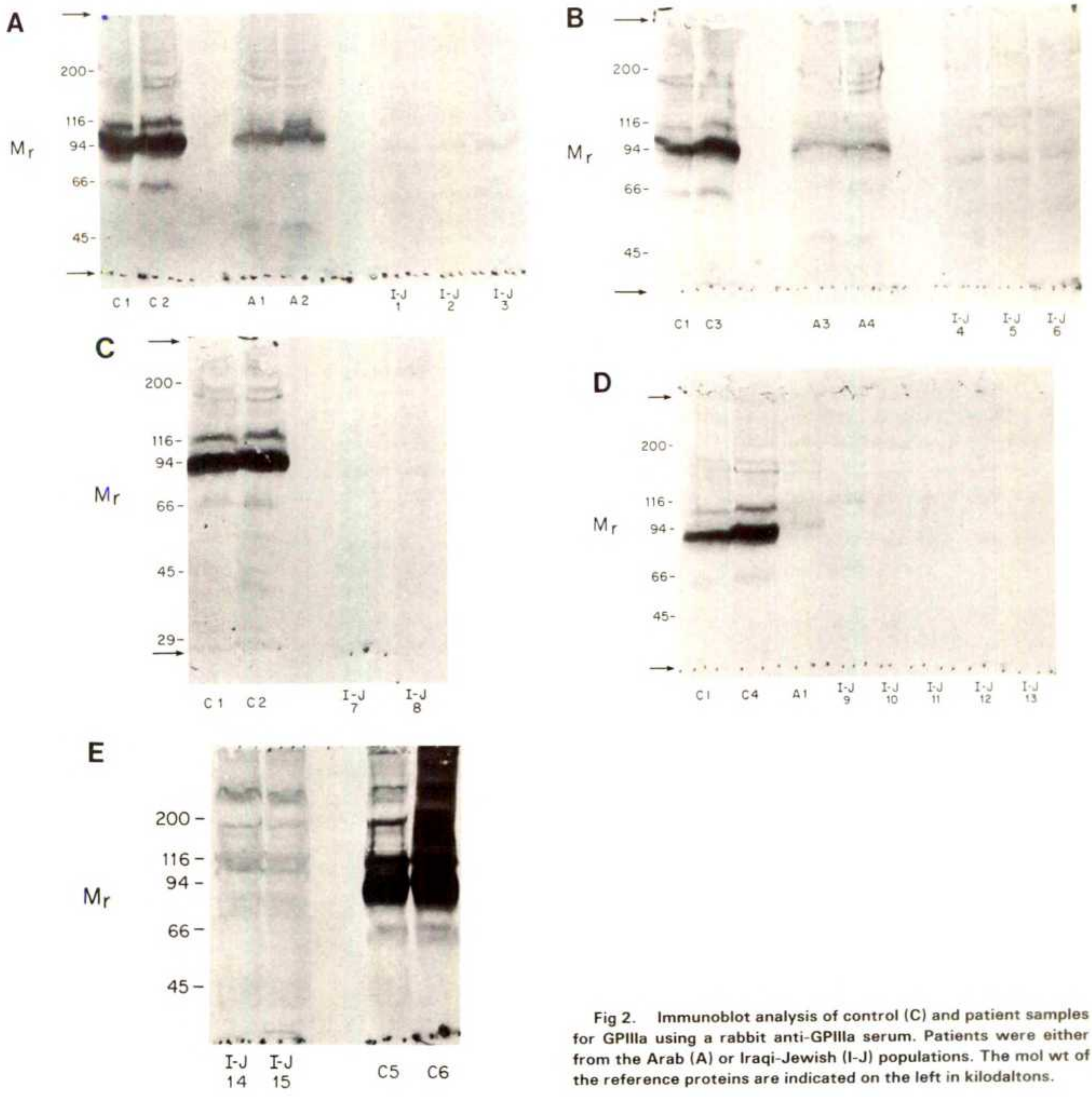

Fig 2. Immunoblot analysis of control (C) and patient samples for GPIIIa using a rabbit anti-GPIIIa serum. Patients were either from the Arab (A) or Iraqi-Jewish (I-J) populations. The mol wt of the reference proteins are indicated on the left in kilodaltons.

and 4 were prepared in Israel. All of the controls had the same pattern as that described above, although band 3 was resolved to variable degrees. The samples from all four Arab patients gave a pattern that was distinctly different from the control, but very similar with one another. Band 1 was reduced in intensity and more diffuse, being nearly undetectable on some blots but not others (eg, A-1 in panel A of Fig 2 *v* A-1 in panel D); band 2 was markedly reduced in intensity but still easily visible; and bands 3 and 4 were markedly reduced, being barely visible. Moreover, there appeared to be an additional or enhanced band at mol wt ~47,000 in all four Arab samples.

In contrast to the Arab samples, band 2—the intensely staining mol wt-90,000 protein—could not be detected in the platelet samples obtained from any of the 15 Iraqi-Jewish patients tested. Band 4 also seemed to be missing from all but one (I-J 9) of the Iraqi-Jewish samples. The other two minor bands were either reduced or absent in the platelets from these 14 patients, but the extent of their deficiency was hard to judge because of the variable intensity of nonspecific bands (ie, bands that were also seen with normal rabbit serum) in these regions. One patient (I-J 9), who was unrelated to the other Iraqi-Jewish patients and whose last platelet transfusion was ~1 week before this sample was obtained, had a unique pattern consisting of a reduced but clearly detectable band 1; the absence of both band 2 and band 3; and a slightly reduced but still detectable band 4. This same pattern was obtained when this patient's platelet sample was analyzed a second time and with a second sample obtained ~1 month after a platelet transfusion.

DISCUSSION

A prolonged bleeding time, normal platelet count, absence of adhesion to glass, and absence of the primary wave of ADP-induced platelet aggregation are some of the hallmarks of Glanzmann thrombasthenia.[1-3] The uniformity of these findings, however, belies the extraordinary clinical heterogeneity of the disorder.[1,2,31] Although subtle variability even in the hallmark criteria were recognized in early studies,[1] more dramatic variability was identified in the deficiencies in clot retraction, platelet fibrinogen content, and fibrinogen binding to ADP-activated platelets.[1-3,14,15,24,40,41] This led to attempts to categorize the disorder as type I (absent clot retraction, marked fibrinogen deficiency, and virtually absent fibrinogen binding) or type II (decreased clot retraction, mild or no fibrinogen deficiency, and normal or only slightly reduced fibrinogen binding),[15,40] but it was not clear whether there was a correlation between the clinical symptoms and these laboratory findings.

The discovery that thrombasthenic platelets are deficient in glycoproteins IIb and IIIa,[9,10] and that these glycoproteins exist as a complex to which fibrinogen binds when platelets are activated,[11] allowed for more detailed biochemical analysis of the platelet defects. A variety of techniques have been applied to separating and identifying these platelet proteins,[19,35,42,43] including ones that utilize antibodies directed against either the whole proteins or against Pl^{A1}, an antigen localized to GPIIIa.[35,44-46] Monoclonal antibody binding to intact platelets has also been employed.[11,27,28,47,48]

These techniques were able to identify a subpopulation of patients who had reduced but still readily detectable levels of GPIIb and GPIIIa; quantitative estimates suggested that such patients had anywhere from a trace amount up to 50% of the normal amount of the glycoproteins.[16,18-22,24,26,41,48,49] The laboratory data from several such patients indicated a type II pattern,[18,38] but other patients were found to be discordant with regard to the type II criteria. For example, individual patients have been described who have (1) a severe deficiency of platelet fibrinogen despite having considerable amounts of residual GPIIb and GPIIIa,[25] (2) considerable blood clot retraction despite markedly reduced platelet fibrinogen and undetectable amounts of the GPIIb/IIIa complex,[50] and (3) nearly normal amounts of platelet fibrinogen despite having markedly reduced or undetectable levels of GPIIb and GPIIIa.[23,41] Thus the group designated originally as type II is markedly heterogeneous at the biochemical level, and this complexity is compounded by the discovery of unusual variant patients who have many of the laboratory and clinical manifestations of Glanzmann thrombasthenia but normal or nearly normal amounts of GPIIb and GPIIIa.[19]

The patients who meet the laboratory criteria for type I thrombasthenia also appear to be heterogeneous at the biochemical level. Considerable confusion surrounds whether or not such patients have totally absent or only markedly reduced levels of the glycoproteins. This is not surprising given that different investigators studied different patients and that the methods used to detect the glycoproteins differ considerably in sensitivity. It is extremely important to determine whether the platelets of such patients have even small amounts of the glycoproteins, since this has important implications for the genetics and cellular biology of the disorder. Among the gel electrophoresis techniques, the nonreduced-reduced and two-dimensional gel techniques provide more precise localization of given glycoproteins than does the one-dimensional technique and thus overcome the problem of overlapping proteins to a large extent. Since they are standardized with normal proteins, however, they may not be able to detect abnormal forms of the protein that migrate to other positions. Radiolabeling clearly increases the sensitivity of detection when compared to staining techniques, but such studies need to be interpreted in the light of the labeling's dependence on specific chemical structure that may be abnormal in the disease states. In particular, since some thrombasthenic patients may have a generalized abnormality in the attachment of carbohydrates to platelet glycoproteins,[21] techniques that label carbohydrate structures may be misleading. Moreover, most labeling studies are performed with intact platelets using membrane-impermeant reagents so that only the surface glycoproteins are labeled. Such techniques would not detect abnormal glycoproteins that cannot insert into the plasma membrane, as has been reported to occur in at least one patient with thrombasthenia.[22]

The agarose gel immunologic techniques that employ heterologous antisera have focused on the arc corresponding to the glycoprotein IIb/IIIa complex; the absence of either glycoprotein would therefore result in the absence of this arc, regardless of how much of the other glycoprotein was produced. Although coordinate synthesis of GPIIb and GPIIIa has been assumed to occur under normal conditions, data suggest that in some patients with thrombasthenia the synthesis and/or surface expression may not be coordinate.[22,24,27] Thus the absence of a GPIIb/IIIa complex are cannot be taken as evidence for the absence of both glycoproteins. The binding of radiolabeled MoAbs to intact platelets[28,47,48] must be interpreted not only with regard to the specificity of the antibody and the surface expression of the glycoprotein, but also the ability to determine whether a given low level of binding to platelets is significantly increased above background.

The immunoblot technique, combining the resolution of SDS-PAGE and the detection capabilities of polyclonal antibodies to purified GPIIIa is probably the most sensitive technique currently available; it does, however, depend on the antigenicity surviving solubilization in SDS as well as the abnormal protein sharing at least some immunologic determinants with the normal protein. Assuming that SDS solubilizes essentially all of the platelet proteins, it has the added advantage of measuring the total amount of the glycoproteins, not just that present on the platelet surface or that which can be solubilized in Triton X-100. Nurden et al[27] studied seven unrelated patients who met the laboratory criteria for type I thrombasthenia and two patients who met the criteria for type II with this technique, using radiolabeled protein A to detect the immunoreactive bands. They estimated the sensitivity of their immunoblots at 1% for GPIIb and 0.5% for GPIIIa. The two type II patients, who previously were shown to have 13% and 15% of normal GPIIb/IIIa complex by crossed-immunoelectrophoreses, had GPIIb and GPIIIa bands that were only moderately decreased in intensity when compared to normal. The type I patients were heterogeneous: three patients had weak GPIIb bands and strong GPIIIa bands; three patients had no detectable GPIIb bands and reduced but detectable GPIIIa bands; and in only one patient was there no detectable GPIIb and GPIIIa.

In the present study we have applied a modified immunoblot technique for GPIIIa to the thrombasthenic patients in Israel who come primarily from the Iraqi-Jewish and Arab populations. All of these patients were studied previously and found to have laboratory evidence of type I thrombasthenia.[28,31]

Our antibody to GPIIIa identified a major band at mol wt 90,000, which corresponds in mol wt to purified GPIIIa. In addition, however, there were minor bands at mol wt ~110,000, 81,000, and 64,000. The mol wt 110,000 and 81,000 bands were also identified by a human antibody to the Pl^{A1} antigen (known to be present on GPIIIa)[35] and a murine MoAb to GPIIIa, suggesting that they are related to GPIIIa rather than being due to contamination of the original antigen with additional platelet proteins.

After the present studies were completed, Dancis et al independently reported on the characterization of the monoclonal anti-GPIIIa and the human anti-Pl^{A1} antibodies used in this study.[51] They found that both antibodies reacted in immunoblots of normal platelet membranes with a major band at mol wt 100,000 (comparable to our band 2, mol wt 90,000), a band 22,000 higher in mol wt (comparable to our band 1, mol wt 110,000), and a lower band of mol wt 71,000 (comparable to our band 4, mol wt 64,000). They further showed that these bands are immunologically related because a similar MoAb lost reactivity against all three bands when adsorbed with the major band and that an affinity column containing the MoAb used in this study immunopurified both the mol wt 100,000 and mol wt 122,000 proteins. They did not identify a band comparable to our band 3, but this may be related to minor technical differences, since we found that this band was difficult to resolve from the heavy, and broad band 2. It is not clear why they were able to identify the mol wt-71,000 band with these antibodies, whereas we were only able to identify a similar band with our heterologous antiserum. If this band is a proteolytic fragment of GPIIIa, it is possible that this is a matter of sensitivity, since they used platelet membrane preparations that may have been more likely to have undergone proteolysis during preparation than the whole platelet samples that we employed. In fact, chymotrypsin digestion of GPIIIa has been reported to result in the production of fragments of mol wt 58,000 to 60,000 that react with anti-Pl^{A1} sera[52,53] and may be comparable to these lower mol wt bands. It will remain for more detailed biochemical analyses of these minor bands to establish their relationship, if any, with GPIIIa. Interestingly, the minor bands observed by Dancis et al[51] and in the present study have not been reported before in immunoblotting studies of GPIIIa using either anti-GPIIIa or anti-Pl^{A1} sera.[27,52,53] The technique we used, which employs a gold-labeled second antibody and a subsequent silver enhancing step, is said by the manufacturer to be more sensitive than other methods, and this may account for these minor bands being detectable. In fact, we assessed the sensitivity for GPIIIa to be at least fivefold greater than that reported by Nurden.[19]

It is tempting to speculate that the mol wt-110,000 band is a precursor of GPIIIa, but, in fact, post-translational changes in carbohydrate (or other moieties) or modifications in the disulfide bond formation may account for the higher apparent mol wt. Moreover, Kieffer et al[52] found that a human anti-Pl^{A1} serum that reacted only with a mol wt-89,000 GPIIIa band on native platelets reacted with a mol wt-105,000 protein when platelets were treated with chymotrypsin; they speculated that the limited proteolysis may have altered the conformation of the molecule in a manner similar to that produced by disulfide bond reduction, which is known to increase the apparent mol wt of GPIIIa in SDS-PAGE. Thus if a low level of endogenous protease activity exists, perhaps a minor component of higher apparent mol wt could be generated.

Our studies were aided by having a large number of patients from the Iraqi-Jewish population in which generations of intramarriage make it likely that most patients share the same genetic defect. This allowed us to analyze the consistency of immunoblot patterns in such a population. In fact, 14 of the 15 patients studied from this population had the same immunoblot pattern: no detectable major band and either reduced or absent minor bands. Thus none of the techniques used to study these patients have been able to clearly identify any residual GPIIb or GPIIIa, leaving open the possibility that these patients suffer from a deletion of genetic information.

The one Iraqi-Jewish patient who differed from the rest of this group had a unique pattern: absent mol wt-90,000 and mol wt-81,000 bands and reduced but clearly detectable mol wt-110,000 and mol wt-64,000 bands. Although it is possible to propose models that might account for such a finding and still be consistent with all of the protein bands being related to GPIIIa, more information is required before a plausible model can be proposed. Since his last platelet transfusion was ~1 month before one of the samples was obtained, it is unlikely that any of the transfused platelets remained in his circulation. The presence of only the mol wt-110,000 and mol wt-64,000 bands argues against these bands being the result of minor in vitro proteolysis of the mol wt-90,000 band. It is interesting to speculate on the genetics in this patient and how they may differ from the genetics in the other Iraqi-Jewish patients. In particular, since the frequency of the carrier state of the commoner defect is so high in this population,[31] it is possible that this patient is doubly heterozygous for two different defects rather than homozygous for a different recessive gene.

The four Arab patients come from four unrelated families currently living in different regions in Israel. It was interesting, therefore, that all four patients tested had the same pattern: reduced but detectable major band, reduced minor bands, and the appearance of a new band at mol wt 47,000. Thus these patients cannot have a total deletion of GPIIIa genetic information. Moreover, given the heterogeneity previously observed in immunoblot studies of type I thrombasthenics,[27] the similarity in patterns in all four patients suggests that they may share the same genetic defect. Although there are many different ways to explain the presence of the additional band, one simple interpretation is that the patients make an abnormal form of the protein that is cleaved by a protease(s) at this site.

The consistency of the immunoblot patterns in Iraqi-Jewish and Arab populations emphasizes the relationship among members of each group and their difference from patients in the other group. In this way it demonstrates the power of the technique to differentiate among patient populations that are identical by all other methods. As DNA probes for these glycoproteins become available, it will be interesting to correlate the immunoblot data with the DNA abnormalities.

NOTE ADDED IN PROOF

Since submission of this manuscript, we have studied another Arab patient with thrombasthenia. The patient is now living in the United States, and the sample was kindly obtained by Dr David Green of Northwestern University (Chicago). As with the other Arab patients, the Mr 90,000 band was markedly reduced, but still readily detectable, indicating the presence of GPIIIa.

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Level of Protein C Determined by Combined Assays During Disseminated Intravascular Coagulation and Oral Anticoagulation

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We have developed a variation of the solid-phase enzyme-linked immunosorbent assay (ELISA) to enable measurement of the activity and antigen levels of protein C (PC) in human plasma. With this assay it is possible to do both tests with the same sample and same microtiter plate coated with anti-PC monoclonal antibody (MCA)JTC-4, which inhibited neither activation of PC nor activity of activated PC (APC). Even in patients undergoing heparin treatment for severe disseminated intravascular coagulation, there were no detectable differences between amidolytic activity and antigen levels of PC in patients' plasma. In addition, there was a strong correlation between the immunologic levels of PC in patients' plasma determined both by polyclonal ELISA using peroxidase-labeled immunopurified antiprotein C-IgG and those found with MCA ELISA using peroxidase-labeled MCAJTC-5, which does not bind to APC. In contrast, when oral anticoagulation therapy was started, immunologic levels of plasma PC estimated by peroxidase-labeled MCAJTC-1, a MCA that recognizes a γ-carboxyglutamic acid domain-related conformational change of PC induced by metal ions, decreased more rapidly than did either the PC level determined by polyclonal ELISA or the percent prothrombin time. This suggested that comparison of MCAJTC-1-recognized PC levels and prothrombin time may be necessary at the beginning of oral anticoagulation therapy to treat patients safely.

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PROTEIN C (PC) is a vitamin K-dependent proenzyme of a serine protease that exerts an anticoagulant activity through selective inactivation of factor Va and VIIIc.[1,2] In addition, activated PC (APC) induces a fibrinolytic state when infused into dogs[3] and decreases plasminogen activator inhibitor activity produced by cultured bovine aortic endothelial cells when cells are treated with APC.[4]

Clinical evidence for PC's role as a major antithrombotic factor arose from the observation that homozygous PC deficiency led to fatal neonatal purpura fulminans.[5] Although many assays have been developed to measure PC activity and antigen levels,[6-10] they all quantitate these two parameters separately.

Since this could be one reason that previous reports[10-12] showed differences between PC activity and antigen levels in patients with disseminated intravascular coagulation (DIC), we developed a variation of ELISA to measure the levels of both PC activity and PC antigen in the same sample using one polystylene plate coated with anti-PC MCA. By using anti-PC MCA specific to the Ca^{2+}-induced PC conformation, the effects of warfarin treatment on the γ-carboxyglutamic acid (Gla) domain of PC were explored.

MATERIALS AND METHODS

Reagents. The following were purchased: essentially fatty acid-free bovine albumin; heparin sodium salt from porcine intestinal mucosa (170 μ/mg); horseradish peroxidase (type IV); Tween 20 (Sigma, St. Louis); solid-state lactoperoxidase-glucose oxidase; horseradish peroxidase-conjugated goat antimouse immunoglobulin (Bio-Rad, Richmond, CA); Sephadex G-25, Sephadex G-200, CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden); (DEAE diethyl aminoethyl)-cellulose (Whatman, Maidstone, Kent, England); 2,2'-azino-di(3-ethyl-benzthiazoline sulfonate); ABTS; (Kirkegaard & Perry, Gaithersburg, MD); H-D-Glu-Pro-Arg-p-nitroanilide (S2366) was a kind gift from Daiichi Chemical (Tokyo). Thrombin was purified by the method of Lundblad et al.[13] Human protein C was purified from citrated fresh human plasma as described by Suzuki et al.[14] The synthetic peptide Asp-Pro-Glu-Asp-Gln-Glu-Asp-Gln-Val-Asp-Pro-Arg-Leu-Ile-Asp-Cys was kindly given to us by Dr J. Stenflo, Malmo, Sweden. Thrombomodulin was purified from rabbit lung extract according to Esmon et al.[15] Human antithrombin III was isolated as described previously.[16] The extinction coefficients and mol wt used for calculating protein concentration were protein C, $E^{1cm}_{1\%} = 14.5$, 62,000; thrombin, $E^{1cm}_{1\%} = 18.3$, 37,000; mouse immunoglobulin, $E^{1cm}_{1\%} = 15.5$, 150,000. Monospecific antiserum against human protein C was prepared in rabbits as described.[17] Monoclonal antibodies (MoAbs) were produced according to the standard method as described previously[17] using BALB/c mice and the mouse myeloma cell line P3-X63-Ag8-U1 (P3U1).

Serum and ascites immunoglobulin fractions used in this study were purified by DEAE-cellulose and protein C-sepharose affinity chromatography. Enzyme-coupled antibodies were prepared by coupling horseradish peroxidase using meta-periodate according to the method of Nakane and Kawaoi.[18] Conjugated enzyme was separated from unconjugated immunoglobulin and enzyme by gel filtration on a Sephadex G-200 column and was then stored at −20°C until use.

Immunoblotting technique. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels was carried out using a resolving gel of 10% acrylamide and a stacking gel of 4% according to the method of Laemmli.[19] After fractionation by SDS-PAGE, the proteins in the gel were electrophoretically transferred (50 V, 1.5 hours) to nitrocellulose (Bio-Rad) using an electroblot apparatus (Marysol Industry, Tokyo) with a cooling system and were subjected to immunoblotting analysis as described previously.[17]

Activation of protein C. PC (720 μg/mL) was activated with thrombin (13 μg/mL) and thrombomodulin (7 μg/mL) in 0.05 mol/L Tris-HCl, pH 7.4, 0.14 mol/L NaCl (TBS) containing 2.5 mmol/L Ca^{2+} at 25°C as described previously.[17] APC was separated from thrombin, thrombomodulin, and residual PC by MCAJTC-1 and MCAJTC-5 immobilized on sepharose as described in Results.

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Radioiodination. PC and MoAbs were radioiodinated using lactoperoxidase-glucose oxidase and Na ^{125}I (19 Ci/mg) (New England Nuclear, Boston). The labeled PC and MCAJTC-1, MCAJTC-4, and polyclonal antibodies had specific activities of 9 × 10^4, 6 × 10^5, 6.6 × 10^5, 7.9 × 10^5 cpm/µg respectively.

Competition for MCAJTC-5 between protein C and synthetic activation peptide of protein C. Polyvinyl chloride microtiter plates were coated with MCAJTC-5 at 10 µg/mL in 0.05 mol/L carbonate buffer, pH 9.6, for 16 hours at 4°C. The plates were then washed several times with TBS containing 1% bovine serum albumin. Complete coating of the well surface was accomplished by incubation with the same buffer for one hour at 25°C. After washing three times with TBS containing 0.05% Tween 20 (TBS-Tween), ^{125}I-labeled PC was added to the well and the subsaturating level of PC was determined. Various concentrations of activation peptide and a constant subsaturating level of PC were added simultaneously to plates coated with MCAJTC-5 and were incubated for three hours at 37°C. After washing five times with TBS containing 0.05% Tween 20, the wells were removed and the radioactivity in each well was determined.

Binding studies. The dissociation constants (Kd) of ^{125}I-labeled MCAJTC-4 for human PC and APC were assessed by a solid-phase assay, previously described by Frankel and Gerhard.[20] Polyvinyl chloride microtiter plates (96 wells, Titer Tek; Flow Laboratories, McLean, VA) were coated with PC or APC dissolved at a concentration of 10 µg/mL in TBS for 16 hours at 4°C. After being washed five times with TBS containing 1% bovine serum albumin and 0.05% Tween 20, the plates were incubated with TBS containing 1% bovine serum albumin for two hours at 37°C. Various concentrations of ^{125}I-labeled antibody, dissolved in TBS, were added to the coated wells and allowed to incubate for three hours at 37°C. The solution was removed by aspiration, and the wells were washed three times with TBS containing 1% bovine serum albumin and 0.05% Tween 20. After the final wash the wells were individually removed and subjected to γ counting. For each antibody concentration the results of four replicate wells were averaged. Subtracted from these counts were those from controls in which the same concentration of ^{125}I-labeled antibody was incubated in wells fully coated with albumin. The level of the radioactivity found in each well was converted to bound antibody concentration, and the dissociation constants were calculated as previously described.[17]

Amidolytic assay. Polyvinyl chloride microtiter plates were coated with MCAJTC-4 (10 µg/mL) in the same manner as in the competition study. After washing five times with TBS-Tween, purified PC, normal plasma, or patients' plasma in washing buffer (100 µL/well) were added to the coated wells and incubated for four hours at 37°C. The wells were then washed five times with TBS-Tween. A mixture of α-thrombin (4 ng/mL) and different concentrations of thrombomodulin, dissolved in TBS-Tween containing 1% bovine serum albumin and 5 mmol/L calcium chloride, was added to each well and incubated for various times at 37°C. After washing three times with TBS-Tween, antithrombin III (250 µg/mL), dissolved in TBS containing 1 U/mL heparin, was added to each well and incubated for 30 minutes at 25°C. H-D-Glu-Pro-Arg-P-nitroanilide (S2366) was then placed in each well. Hydrolysis was performed at 25°C, and the absorbance at 405 nm was monitored on an ELISA analyzer ETY-96 (Toyo Sokki, Tokyo).

Solid-phase ELISA. Levels of PC antigen binding to MCAJTC-4-coated wells were determined before and after activation with thrombin-thrombomodulin complex. Before activation with thrombin-thrombomodulin, peroxidase-conjugated PCA, MCAJTC-5, or JTC-3 in TBS-Tween was added to each well in the absence or presence of 5 mmol/L $CaCl_2$ and were then incubated at 4°C for 16 hours. At the end of this period, after washing three times with TBS-Tween with or without $CaCl_2$, 200 µL of freshly mixed ABTS was placed in each well according to the manufacturer's directions. Hydrolysis was monitored at 25°C on an ELISA analyzer at 405 nm. For sequential determination of PC amidolytic activity and antigen levels bound in the same well, each well was washed three times with TBS-Tween after the APC amidolytic activity had been measured, and then horseradish peroxidase-conjugated antibody was added. Standard curves for each assay were constructed by using purified human PC dissolved in TBS containing 1% bovine serum albumin or either PC-deficient plasma or PC-immunodepleted plasma supplemented with purified human PC.

Collection of plasma samples. Blood was collected from antecubital veins into 0.1 vol of 3.8% sodium citrate and was centrifuged at 2,000 g for 20 minutes to prepare platelet-poor plasma (PPP). Pooled normal PPP was obtained from 20 healthy donors. All plasma was stored at −80°C until use. Single time-point plasma samples were taken from patients with DIC, which had been confirmed by marked reductions in platelet counts, fibrinogen, antithrombin III (except in acute promyelocytic leukemia), plasminogen, and α_2-plasmin inhibitor levels, and prolonged partial thromboplastin and prothrombin times. All patients had elevated levels of fibrin degradation products and of fibrinopeptide A release, as measured by radioimmunoassay (RIA) described by Nossel et al.[21] Patients with pre-existing liver disease or laboratory evidence of liver disease were eliminated from the DIC group in this study. Patients with laboratory evidence of DIC in association with acute leukemias (five acute promyelocytic, ten myeloid, and two lymphoid) and 16 other clinical conditions (septic, carcinomatous, or obstetric) were investigated. In the sequential study, four serial samples were taken over a period of time from five patients, including one associated with acute promyelocytic leukemia. Patients with severe chronic liver disease, except for hepatoma, had laboratory data consistent with liver failure (albumin <2 g/100 mL, cholinesterase <0.2 pH/hour, elevated transaminases, and prolonged prothrombin time). Plasma samples from previously described congenital PC-deficient patients[17,22] and their families with no thrombosis for a period of 6 months were analyzed. Patients starting warfarin therapy due to deep-vein thrombosis or myocardial infarction were treated without a loading dose and received the same dose of warfarin for several days prior to testing.

RESULTS

The antigenic target for MCAJTC-4 and MCAJTC-5, two antibodies that have no effect on APC amidolytic activity, is the PC heavy chain, as has been described.[17] As shown in Fig 1, MCAJTC-4 bound to both PC and APC, but MCAJTC-5 did not bind to APC. An experiment was designed to test whether ^{125}I-labeled APC could bind to microtiter plates coated with MCAJTC-5. Prior to our binding experiments, the PC subsaturating level was determined using ^{125}I-labeled PC. In the binding experiment, ^{125}I-labeled PC was activated at 25°C with thrombin and thrombomodulin as described.[17] After incubation for various time periods (Fig 2), samples were removed and mixed with antithrombin III to terminate the activation. One portion of each sample was used to determine APC activity against S2366, while the remainder was added to individual wells of a plate coated with MCAJTC-5 to observe binding of the radioactivity. Figure 2 shows that as APC amidolytic activity increased in each sample, the binding of radioactivity to the plate gradually decreased.

During the activation of human PC by thrombin-thrombomodulin complex, an Arg_{12}-Leu_{13} bond is cleaved at the

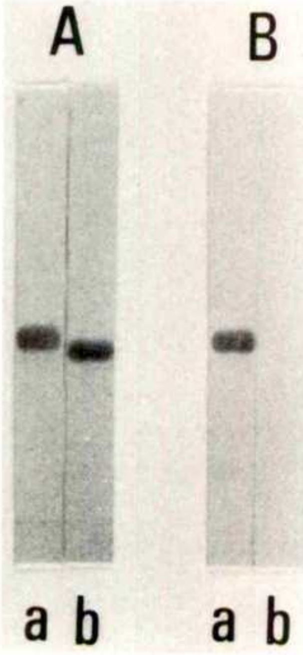

Fig 1. Immunoblotting of human PC and APC after SDS-PAGE using MoAbs against human PC. Five hundred nanograms of human PC (a) or APC (b) were electrophoresed by SDS-PAGE, and the protein was transferred to a nitrocellulose membrane. Strips cut from the membrane were incubated with MoAbs JTC-4 (A) or JTC-5 (B). Immunoreactive protein bands on these strips were detected by peroxidase-conjugated goat antimouse IgG immunoglobulin and 4-chloro-l-napthol as described in the Materials and Methods section.

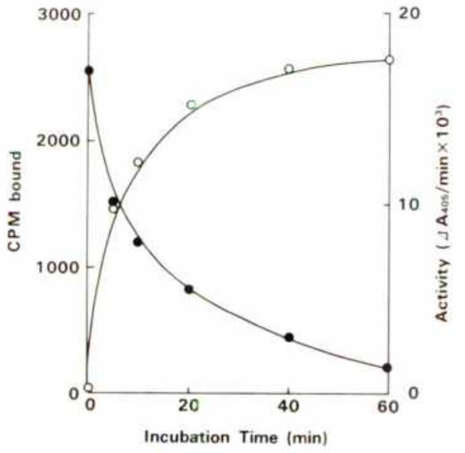

Fig 2. Effect of PC activation on PC binding to MCAJTC-5. ^{125}I-labeled PC (720 μg/mL) was activated by thrombin (13 μg/mL) and thrombomodulin (7 μg/mL) in a tube at 25°C. After various lengths of time, aliquots of the mixture were removed, and activation was terminated by the addition of antithrombin III (2 mg/mL). The APC present was measured by hydrolysis of S2366. A constant and subsaturating level of the PC and APC mixture was added to individual wells coated with MCAJTC-5 at each time. The binding of PC to MCAJTC-5 was evaluated by measuring radioactivity in each well.

amino-terminal end of the protein's heavy chain, releasing a small dodecapeptide of mol wt 1,400.[23] To determine whether the MCAJTC-5 had an epitope in the activation peptide region, a competitive binding assay was carried out using ^{125}I-labeled PC and a synthetic dodecapeptide.

The binding of ^{125}I-labeled PC to MCAJTC-5 was diminished by the presence of the synthetic dodecapeptide in a dose-dependent manner (Fig 3). However, a concentration of dodecapeptide higher than that of PC was necessary to prevent the binding of MCAJTC-5 to PC due to the lower affinity of MCAJTC-5 to dodecapeptide (Kd = 9.6×10^{-8} mol/L^{-1}).

Moreover, MCAJTC-5 inhibited the activation of PC both by thrombin and thrombin-thrombomodulin (data not shown). These results indicate that MCAJTC-5 recognizes the activation peptide region of PC. A similar MoAb against the PC activation-peptide region was previously reported by Laurell et al.[24]

The dissociation constants for ^{125}I-labeled MCAJTC-4 and MCAJTC-5 to human PC and APC were determined by the assay described in the Materials and Methods section. PC was activated by thrombin-thrombomodulin complex in the presence of Ca^{2+}.[17] After activation, the complex was removed by the application of the activation mixture to MCAJTC-1 immobilized on a column. PC and APC bound to this in the presence of Ca^{2+} and were eluted in its absence.[17] Trace amounts of residual PC were removed from the eluate using MCAJTC-5 immobilized on a column. Pure APC was obtained in the breakthrough. Prior to the binding experiments were confirmed by a competition assay that the affinities of radiolabeled MCAJTC-4 and MCAJTC-5 for PC and APC were the same as those of the native nonlabeled MoAbs. The data were treated by a modified Scatchard plot, as described by Frankel and Gerhard.[20] Each Sips heterogeneity index, α, for JTC-4 and JTC-5 toward PC and APC, when calculated from the data approximated 1.0. The dissociation constants for JTC-4 with PC (Kd = 6.80×10^{-9} M) and APC (Kd = 8.70×10^{-9} mol/L) were very close (Table

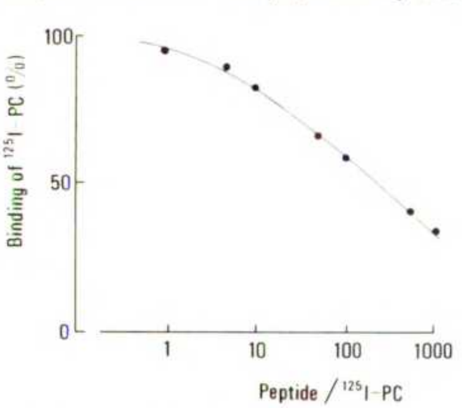

Fig 3. Competitive binding of PC and synthetic dodecapeptide to JTC-5. A constant and subsaturating level of ^{125}I-labeled PC for MCAJTC-5 was mixed with various concentrations of synthetic dodecapeptide and added to individual wells of plate coated with 10 μg/mL MCAJTC-5. The results are shown as a percentage of ^{125}I-labeled PC bound in the absence of competing synthetic peptide.

Table 1. Dissociation Constants for Anti-PC Antibodies to PC and APC

	Kd	
IgG	Protein C	Activated Protein C
JTC-4	6.86×10^{-9} M	8.70×10^{-9}
JTC-5	5.10×10^{-9}	ND*

For determination of the dissociation constant (Kd), a variation of Scatchard analysis using ^{125}I-labeled MoAb was performed as described in the Materials and Methods section.

*MCAJTC-5 did not bind to APC.

1), but ^{125}I-labeled MCAJTC-5 showed no binding to solid-phase APC.

PC antigen levels were determined by ELISA in purified preparations and in PC-deficient plasma supplemented with PC using a combination of JTC-4 and horseradish peroxidase-coupled immunopurified anti-PC polyclonal antibody. A linear dose response curve of PC concentration against absorbance at 405 nm was obtained (data not shown). When a standard curve was constructed using plasma reconstituted by mixing normal and PC-immunodepleted plasmas, the results were also satisfactory (data now shown). Because the dissociation constants for MCAJTC-4 with PC and APC were very close, we explored the possibility of a combined assay system for PC antigen and PC function in plasma samples.

Figure 4 shows the activation of PC bound to JTC-4 coated wells with a mixture containing 4 ng/mL of thrombin and different amounts of thrombomodulin. Since more than 10 ng/mL of thrombomodulin did not increase further the activation rate of PC and maximum activation was obtained after one hour of incubation at 37°C in the presence of 5 mmol/L $CaCl_2$, these conditions were chosen for routine activation. After determination of the level of APC activity, plates were washed and the APC antigen level was measured. As shown in Fig 5, the observed levels of PC and APC antigens correlated well, and the between-assay coefficient of variation was 2.5%. Furthermore, good correlation was observed between PC levels determined by this assay and by the Laurell rocket technique[25] (data not shown). To determine whether this assay could detect both plasma PC activity and antigen levels, PC-deficient plasma supplemented with human PC was used to construct standard curves (Fig 6). A linear relationship between concentrations of PC added and of synthetic substrate cleaved was observed.

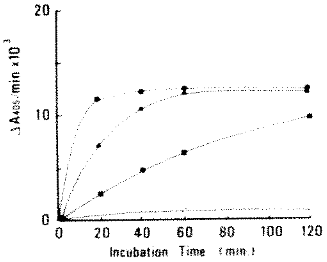

Fig 4. Activation of PC bound to MCAJTC-4 coated wells by thrombin (4 ng/mL) and 0 (○), 2 (■), 5 (▲), and 10 (●) ng thrombomodulin/mL in the presence of 5 mmol/L $CaC1_2$. APC was measured by hydrolysis of S2366.

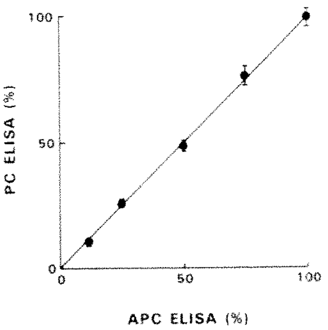

Fig 5. Correlation between PC antigen levels determined by ELISA before and after activation. Experimental conditions were as described in the Materials and Methods section. Vertical bars represent SEM of nine measurements.

Since APC in plasma may exist as a complex with its specific inhibitor,[26,27] the effect of plasma PC inhibitor (PCI) on the binding of APC to MCAJTC-4 was tested. To remove the effect of PC in normal plasma on the binding of APC to the plate, citrated and heparinized PC-deficient plasma were used, since these plasma inhibited APC activity in the same way as normal plasma, as previously reported by Comp et al.[7] These plasma samples were preincubated for 60 minutes at 37°C with various concentrations of APC and were then added to wells coated with a constant amount of MCAJTC-4. The concentrations of APC and APC-PCI complex bound to MCAJTC-4 were determined by polyclonal ELISA (a combination of MCAJTC-4 and peroxidase-conjugated polyclonal anti-PC).

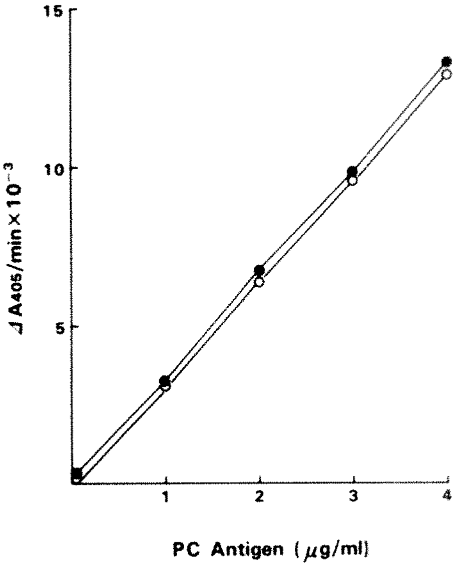

Fig 6. Effect of PC concentration on amidolytic activity. Purified human PC dissolved in TBS containing 1% bovine serum albumin (●) or PC-deficient plasma supplemented with purified PC (○) was added to wells of plates coated with MCAJTC-4. After washing, PC bound to the wells was activated by thrombin and thrombomodulin. Amidolytic activity of APC bound to the wells was measured as described in the Materials and Methods section.

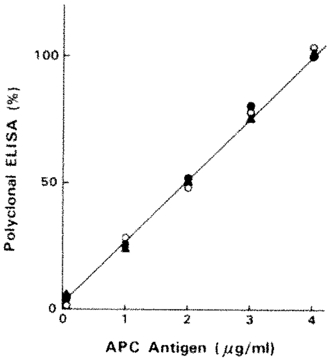

Fig 7. Effect of plasma PC inhibitor on the binding of APC to MCAJTC-4. Various concentrations of APC as indicated were preincubated with TBS containing 1% bovine serum albumin (●), congenital PC deficient plasma (O), or PC immunodepleted plasma (▲) for one hour at 37°C. Level of APC antigen was determined as described in the Materials and Methods section.

As shown in Fig 7, it was suggested that MCAJTC-4 recognized APC and APC-PCI complex in a similar way. Figure 8 illustrates the relationship of amidolytic activity to PC antigen levels after activation in controls and in the patient series using MCAJTC-4 and peroxidase-labeled polyclonal anti-PC IgG. The between-assay coefficient of variation was less than 2.3% when purified PC or PC-deficient plasma supplemented with purified PC was used.

There was a good correlation between the PC antigen levels and amidolytic activities in normal subjects, in patients with liver cirrhosis, and in warfarin-treated patients. In those with DIC, the correlation was also satisfactory, although PC levels were decreased (40 ± 21%, n = 27), except when DIC was associated with acute promyelocytic leukemia (90 ± 15%, n = 5). We also analyzed four serial samples from five cases of DIC with or without liver damage. Although PC activity and antigen levels varied according to the course of the DIC, there were no detectable discrepancies between the PC activity and antigen levels in each patient's plasma (data included in Fig 8). Since heparinized normal plasma and PC-deficient plasma did not support the binding of ^{125}I APC to MCAJTC-5 coated wells (data not shown), it was supposed that MCAJTC-5 recognized neither APC (Figs 2 and 3) nor APC-PCI complex.

To confirm that there was almost no discrepancy between PC activity and antigen levels in DIC patients, the patients' PC antigen levels were also measured by ELISA using a combination of MCAJTC-4 and conjugate-labeled MCAJTC-5 (JTC-5 ELISA) or polyclonal anti-PC antibody (polyclonal ELISA). The between-assay coefficient of variation was 1.8% when purified PC or PC-deficient plasma supplemented with purified PC were used. As shown in Fig 9, there were no discrepancies between polyclonal ELISA and JTC-5 ELISA-determined PC antigen levels in samples from 40 patients with DIC.

It was suggested that MCAJTC-1 recognized a Gla domain-related conformational change induced by metal ions, evidenced by the fact that half-maximal binding was observed at a calcium concentration of 0.5 mmol/L, by the fact that the antibody, even in the presence of Ca^{2+}, did not react with Gla domainless PC, and by the fact that Zn^{2+} and Tb^{3+} supported binding in essentially the same way.[17]

Since MCAJTC-4 did not interfere with the binding of MCAJTC-1 to PC in the presence of Ca^{2+},[17] the amount of PC recognized by MCAJTC-1 in disease states and during warfarin therapy was measured using MCAJTC-4-coated

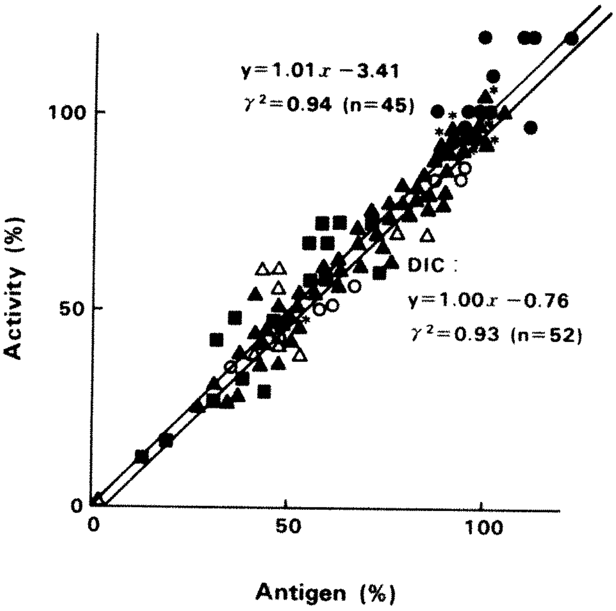

Fig 8. Relationship of PC amidolytic activity to plasma PC antigen levels in normal controls (●), patients with chronic liver disease (■), patients receiving oral anticoagulant therapy (O), patients with PC congenital deficiency (△), and patients with DIC (▲). *▲ - DIC with acute promyelocytic leukemia.

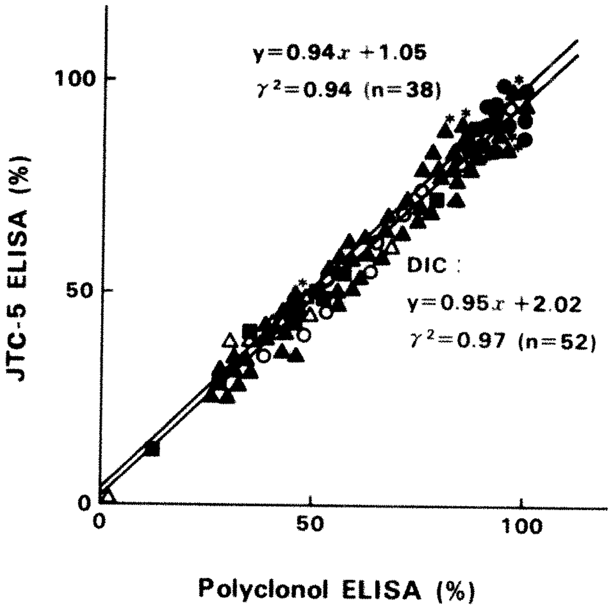

Fig 9. Correlation between the polyclonal ELISA and JTC-5 ELISA. Plates were coated with MCAJTC-4. The level of antigen bound to MCAJTC-4-coated wells was quantitated using peroxidase-conjugated rabbit anti-PC polyclonal antibody (polyclonal ELISA) or peroxidase-conjugated JTC-5 (JTC-5 ELISA). Symbols are the same as in Fig 8.

wells and peroxidase-conjugated MCAJTC-1 and was calculated from a standard curve as percent binding. The between-assay coefficient of variation was 2.3% when purified PC or PC-deficient plasma supplemented with purified PC were used. The ratio of PC antigen determined by JTC-1 ELISA to that measured by polyclonal ELISA in the presence of CA^{2+} was not significantly different from unity in normal controls and in patients with liver cirrhosis and DIC (Fig 10). However, as we previously reported,[17] this ratio was reduced in warfarin-treated patients. Although PC, recognized by MCAJTC-1, might not be necessarily Gla domain-intact PC, to obtain a more convenient assay to measure Gla domain-intact PC in warfarin-treated individuals, the levels of PC in sequential plasma samples taken over a period of time from five patients treated with warfarin without a loading dose were measured by polyclonal, JTC-1, and JTC-5 ELISAs.

In all cases the levels of PC determined by JTC-1 ELISA decreased more rapidly than when measured by polyclonal or JTC-5 ELISA. Moreover, PC antigen levels determined by JTC-1 ELISA were reduced more rapidly than percent of prothrombin time (Fig 11).

DISCUSSION

Previous studies suggested that levels of PC antigen were low in clinical conditions associated with DIC,[10,11,12,28] and increased clearance of in vivo activated PC has been suggested as an explanation. However, Radeghiero et al[29] did not find a significant decrease in PC in their patients with DIC associated with acute leukemia. Our results are similar to theirs only in DIC cases associated with acute promyelocytic leukemia. Such DIC cases may be exceptional because these patients had normal PC and antithrombin III activity and antigen levels, although they had high levels of fibrinopeptide A release and α_2PI-plasmin complex (data not shown). Marlar et al[11] measured serial PC levels during DIC progression and observed that the PC activity decreased in DIC patients in parallel with PC inhibitor (PCI) and was lower than PC antigen levels. To determine chromogenic activity "after activation" with thrombin-thrombomodulin, they removed APC from the plasma by polyclonal anti-PC IgG-Sepharose. However, the dose of heparin used to treat DIC patients is enough to enhance the neutralization of APC activity by plasma PCI[27] during activation with thrombin-thrombomodulin in vitro. Furthermore, other components may have been generated during DIC that influenced PC activation. In addition, they separately measured PC antigen levels in the plasma before activation by a radiolabeled Laurel electroimmunoassay.

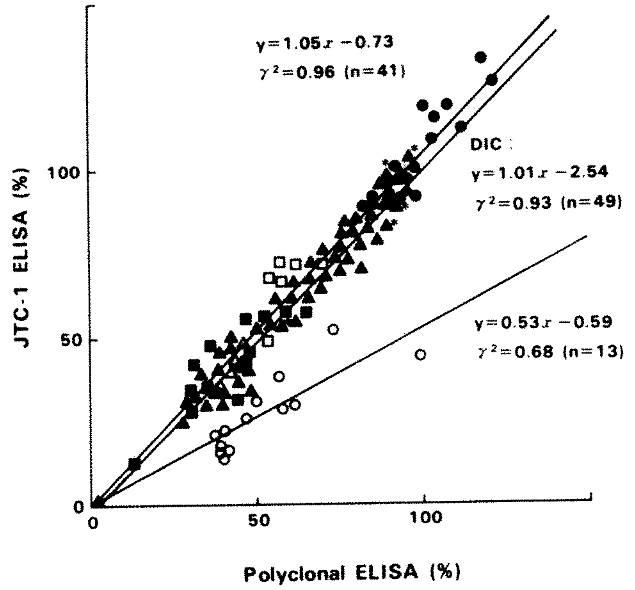

Fig 10. Correlation between polyclonal ELISA and JTC-1 ELISA. Plates were coated with MCAJTC-4. Plasma samples were added to the wells and incubated for three hours at 37°C. After washing, the amount of bound PC was measured in the presence of 5 mmol/L $CaCl_2$ using polyclonal ELISA and peroxidase-conjugated JTC-1 (JTC-1 ELISA). Symbols are the same as in Fig 8.

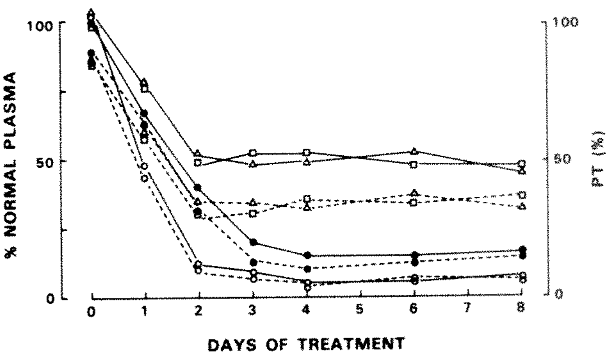

Fig 11. Changes in PC antigen level compared to changes of prothrombin time (●) in plasma of patients with deep vein thrombosis (dotted line) or myocardial infarction (solid line) starting warfarin therapy (10 mgd) without loading dose. Plasma PC antigen level was measured by polyclonal ELISA (□), JTC-5 ELISA (△), or JTC-1 ELISA (○) in the presence of Ca^{2+}.

On the other hand, D'Angelo et al[10] initially removed PC from heparinized plasma in the presence of Ca^{2+} by using a Ca^{2+}-dependent MCA directed against human PC, which bound to human PC independently of the presence of Gla residues. They then determined the activity of the removed PC. They neglected plasma APC and the APC-PCI complex because their Ca^{2+}-dependent MCA recognized neither of them.

However, they could find no discrepancies in the ratio of amidolytic activity in the removed PC to Laurell rocket-determined plasma PC antigen in both controls and DIC patients. In contrast to their separate assays, the combined assay system for PC antigen and activity levels described in this paper provides a suitable method for measuring the specific activity (ratio of amidolytic activity to antigen level) of PC in normal and patient plasma. Before activation we adsorbed PC from plasma to the plate coated with MCAJTC-4, which binds PC, APC, and APC-PCI almost equally. After activation of absorbed PC, thrombin-thrombomodulin was neutralized and washed away. Then the amidolytic and antigen levels of PC just adsorbed on the plate were determined successively. Our results were basically the same as those reported by D'Angelo.

To confirm our results the levels of PC antigen in plasma were determined by polyclonal ELISA without activation (MCAJTC-4-polyclonal anti-PC) and were compared with those determined by JTC-5 ELISA (MCAJTC-4-MCAJTC-5), which was not able to measure APC or APC-PCI. PC levels correlated well in both methods (Fig. 7).

Given that the half-life of APC has previously been reported[30] to average 10 to 15 minutes and that of PC activation peptide five minutes,[12] APC concentrations generated in the systemic circulation of patients with DIC might be as high as 0.02% to 2% of the total circulating zymogen. Our results and previous observations strongly suggest that PC levels are low and APC levels are undetectable in the circulating plasma of individuals with DIC. This may be due to rapid clearance of APC from the plasma by the reticuloendothelial system or due to binding of APC to endothelial cells or other surfaces. However, we cannot deny that there may be detectable APC-PCI complex in the plasma of patients with liver disease-associated DIC due to a decrease in clearance. We also developed the PC assay to enable the assessment of levels of Gla domain-incomplete PC in plasma of patients. This could be achieved by comparison of PC levels determined by JTC-1 ELISA and polyclonal ELISA or JTC-5 ELISA.

Previous studies demonstrated that the Gla domain of PC was essential for optimal PC activation on the endothelial cells and was required for expression of membrane-dependent anticoagulant activity. Recently it has been proposed that the rapid decrease in PC levels after the start of warfarin therapy, relative to the decreases in concentration of most other vitamin K-dependent clotting factors, gives rise to a transient PC deficiency state, resulting in a transient hypercoagulable condition.[31-34]

The levels of PC determined by JTC-1 ELISA may not necessarily correlate with PC anticoagulant activity, since the number of Gla residues in PC required for expression of anticoagulant activity and membrane interaction is not clear. However, the decrease in PC levels measured by JTC-1 ELISA was more rapid than that of percent prothrombin time and of PC levels measured by JTC-5 or polyclonal ELISA (Fig 11). Although no statistical test was applied, these results suggest that comparison of the decrease in PC levels measured by JTC-1 ELISA with that of percent prothrombin time would be useful in recognizing a transient hypercoagulable state in patients treated by oral anticoagulant therapy. Following these observations we treated patients with minidoses of heparin at the beginning of oral anticoagulation therapy, even in cases of nonloading dose treatment. However, since heparin also enhances inhibition of APC by PCI, further studies are needed, especially to treat congenital or acquired PC-deficient patients with warfarin.

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A Novel Platelet Aggregating Factor Found in a Patient With Defective Collagen-Induced Platelet Aggregation and Autoimmune Thrombocytopenia

By Tateo Sugiyama, Minoru Okuma, Fumitaka Ushikubi, Shigeki Sensaki, Kenji Kanaji, and Haruto Uchino

We found a novel platelet aggregating factor in a patient with steroid-responsive immune thrombocytopenic purpura that is associated with defective collagen-induced platelet functions. The aggregating factor and platelet functions were analyzed. The patient, a 58-year-old female, had purpura and prolonged bleeding time despite adequate platelet counts (>140,000/μL) after steroid therapy. The patient's platelets responded normally to all agonists except collagen. Platelet adhesion to collagen fibrils was decreased. The patient's plasma induced irreversible aggregation and ATP release in normal platelet-rich plasma (PRP). This platelet aggregating factor was found in F(ab')$_2$ fragments of the patient's IgG, which caused thromboxane B$_2$ synthesis, elevation of cytoplasmic Ca^{2+} levels, and phosphorylation of 40 kDa protein in normal platelets. Platelet aggregation by the patient's IgG was inhibited by prostacyclin, dibutyryl cAMP, diltiazem, disodium ethylenediaminetetraacetate, and antimycin A plus iodoacetate, but ADP scavengers, cyclo-oxygenase inhibitors, and heparin had little or no effect. The aggregating activity of the patient's IgG absorbed to and eluted from normal platelets. The patient's Fab fragments did not induce platelet aggregation in eight of ten normal PRP but specifically inhibited aggregation induced by collagen and by the patient's IgG. The major component of an immunoprecipitate made with the patient's IgG from radiolabeled membrane proteins of normal platelet extract had a 62 kDa mol wt, while no such precipitate appeared in extracts of the patient's platelets. These results indicated that platelet aggregation by the patient's IgG was induced by the reaction of an antibody with a specific antigen on the normal platelet membrane through stimulus-response coupling. This antigen may be a collagen receptor on the platelet, most likely a polypeptide of 62 kDa under reducing condition. The defect of collagen-induced aggregation of the patient's platelets seemed to be due to alteration of the membrane protein related to this putative collagen receptor.

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IDIOPATHIC thrombocytopenic purpura (ITP) is a common hemorrhagic syndrome characterized by a reduced number of circulating platelets, normal to increased numbers of megakaryocytes, and accelerated platelet destruction.[1,2] There is ample evidence that, at least in adults, platelet destruction is caused by the binding of an antiplatelet autoantibody to platelet-specific antigen. Further implication of ITP as an autoimmune disorder is the strong association between ITP and well-defined autoimmune diseases such as Graves' disease,[3] Hashimoto's thyroiditis,[3] and systemic lupus erythematosus.[4]

Several reports have been published describing an association between antiplatelet antibodies and various platelet function disorders.[5-10] These reports suggest that antiplatelet antibody can contribute to or be responsible for not only quantitative but also qualitative platelet disorders, which can explain why some ITP patients with platelet counts greater than 50,000/μL still present a bleeding tendency. Although these qualitative platelet defects have been attributed to the effect of antiplatelet antibody, the underlying basis for an immune-mediated impairment of platelet functions is still unclear. It may be different from case to case, as recent studies on the antigenic specificity of antiplatelet antibodies in chronic ITP have shown that the target epitope differed among patients.[11] Accordingly, it should be of a great interest to know the nature of the antiplatelet antibody affecting the functions of autologous and/or homologous platelets, especially in relation to the significance of platelet membrane antigen on physiologic platelet responses.

Collagen plays an important role in primary events of hemostasis in vivo.[12] While it can be assumed that there is a specific molecule on the surface of platelets that interacts with collagen, the identity of a collagen receptor on the platelet remains unclear despite extensive investigations in many laboratories.[13-20] In this article we describe a novel platelet aggregating factor found in plasma of an ITP patient who showed a specific defect of collagen-induced platelet aggregation. Analysis of the factor revealed that it has characteristics of an antibody to a putative collagen receptor on the platelet membrane.

MATERIALS AND METHODS

Case report. The patient was a 58-year-old woman in 1986, and her past history revealed that she had given birth to two children without any problem and underwent a cataract operation without excessive bleeding at age 49. There was no family history of bleeding tendencies. In 1982 she was found, during physical examination, to have thrombocytopenia (15,000/μL), but no further examination was undertaken. From September 1983 she suffered from recurrent cutaneous ecchymoses, frequent episodes of epistaxis and gum bleeding, thirst, excessive sweating, palpitation, and weight loss. In December 1983, because of massive nasal bleeding, platelet concentrate prepared from 400-mL blood was transfused at a local hospital, and she was admitted to Kyoto University Hospital with a diagnosis of thrombocytopenic purpura and hyperthyroidism. Physical examination showed multiple ecchymoses and petechiae over the extremities and the trunk as well as diffuse goiter. There was no evidence of

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splenomegaly. Her blood counts showed 326×10^4 RBCs/μL, 6,100 leukocytes/μL and 13,000 platelets/μL. Bleeding time by Duke's method[21] was prolonged (ten minutes; normal < three minutes). A bone marrow aspirate showed hypercellularity with immature megakaryocytes. Prothrombin time, activated partial thromboplastin time, and plasma fibrinogen concentration were all within normal limits. Serum triiodothyronine and total thyroxine levels were elevated, and thyroid-stimulating hormone (TSH) level was decreased. Tests for serum antithyroglobulin and antimicrosome antibodies were positive. A diagnosis of immune thrombocytopenic purpura associated with Graves' disease was made, and treatment with prednisolone (45 mg/d) and methimazole (30 mg/d) was initiated. Her platelet count rose rapidly to 168,000/μL, and prednisolone was tapered to 10 mg/d. Abnormal thyroid test results also quickly improved. Although her platelet counts remained above 140,000/μL (normal range 130,000 to 350,000/μL), occasional ecchymoses and petechiae continued, and bleeding times by Duke's method (five to ten minutes) and a template method[22] (16.5 minutes; normal range three to 9.5 minutes) remained prolonged. Therefore platelet dysfunction was suspected at this time, and we investigated the patient's platelet functions as well as platelet-poor plasma (PPP, vide infra) obtained after the patient's platelet counts became normal. All tests on platelets were done during treatment with prednisolone (5 to 15 mg/d) and methimazole (5 to 10 mg/d) without any other drugs that are known to interfere with platelet functions.

Normal controls. Healthy adult blood donors who had taken no drugs for at least 2 weeks were chosen as normal controls. All studies were done after informed consent was obtained from the patient and normal subjects.

Platelet preparation. The collection of blood and preparation of platelet-rich plasma (PRP) as well as PPP were carried out as previously described.[23] For preparation of washed platelets, PRP was acidified to approximately pH 6.5 with acid citrate dextrose solution (38.1 mmol/L citric acid, 74.8 mmol/L trisodium citrate, 122.1 mmol/L dextrose), and platelets were pelleted by centrifugation at 1,100 g for ten minutes. The platelets were washed twice in platelet washing buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 5 mmol/L glucose, pH 7.4) and finally resuspended in HEPES-buffered saline (10 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na_2HPO_4, 6 mmol/L glucose, pH 7.4) or in buffer, as indicated in each experiment. Formalin-fixed washed platelets (FWP) were prepared according to the method of Kirby and Mills.[24] The FWP were washed twice with platelet washing buffer and resuspended in 10 mmol/L phosphate-buffered saline (PBS), pH 7.4. Platelet counts were done by Thrombocounter C (Coulter Diagnostics, Hialeah, FL). All procedures were carried out at room temperature.

Platelet aggregation and ATP secretion. Platelet aggregation experiments were performed nephelometrically[23] at a density of 3×10^5 platelets/μL on PRP or on washed platelet suspensions using a dual-channel aggregometer (NKK Hematracer, Niko Bioscience, Tokyo, Japan) linked to a pen recorder. Calcium chloride was added to the washed platelet suspension at 1 mmol/L before aggregation studies. In experiments with platelet-inhibiting agents, PRP was preincubated with the inhibitor for three minutes at 37°C before the addition of IgG (100 μg/mL), except for iodoacetate and antimycin A (Boehringer Mannheim, West Germany) with which PRP was preincubated for ten minutes. For the secretion study ATP release was monitored on Lumiaggregometer (Chrono-Log, Havertown, PA), as described previously.[23]

Platelet adhesion to collagen fibrils. Platelet adhesion to collagen fibrils was studied according to a modified method of Mant.[25] In short, blood was collected in one tenth volume of 77 mmol/L EDTA, pH 7.4, and PRP and PPP were prepared as described above. "Acid soluble" collagen (suspension of fine collagen fibrils) was prepared from bovine achilles tendon collagen (type II, Sigma Chemical, St Louis).[25] One fifth milliliter of EDTA-anticoagulated PRP (3×10^5 platelets/μL) was continuously stirred in a cuvette at 37°C on the aggregometer, and collagen at a final concentration of 15, 30, or 60 μg/mL, or saline (as a control) was added. After five minutes, nonadherent platelets in each sample were counted in Thrombocounter C, and platelet adhesion (%) was calculated using the formula: (platelet count of control PRP − platelet count of collagen-treated PRP) × 100/platelet count of control PRP.

Platelet ATP and ADP contents. The platelet contents of ATP and ADP were determined by the firefly-luminescence method as described previously.[23]

Release of lactate dehydrogenase. Samples of PRP (0.2 mL) were stirred at 37°C and exposed to the patient's IgG, collagen, or saline. After five minutes, samples were removed and centrifuged at 10,000 g for two minutes. The lactate dehydrogenase (LDH) activity in the supernatant and its total activity after solubilization with 0.5% (wt/vol) Triton X-100 were determined according to the standard assay technique using an LDH kit (Iatro LDH-LQ, Iatron, Tokyo, Japan). LDH release was expressed by the activity in the supernatant as a percentage of the total activity.

Purification of IgG and preparation of F(ab')$_2$ fragment, Fab fragment, and Fc fragment. Purified IgG was prepared from PPP by affinity chromatography with protein-A Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ).[26] The IgG fraction was dialyzed against PBS and concentrated to the desired protein concentration on Amicon Ultrafiltration Cell (Amicon, Danvers, MA). F(ab')$_2$ fragments were prepared from purified IgG by pepsin digestion[27] and isolated on a Sephadex G100 column (Pharmacia Fine Chemicals). Residual undigested IgG was removed on a protein-A Sepharose column. Fab fragments and Fc fragments were prepared from purified IgG by papain digestion.[28] The digestion products were applied to a Sephadex G100 column to obtain fractions containing Fab fragments and Fc fragments followed by purification of each fragment on a protein-A Sepharose column. Purities of IgG and these fragments were checked by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and by Ouchterlony immunodiffusion. Rabbit anti-κ, anti-λ, anti-Fc (IgG), and polyvalent anti-Ig antisera used were kindly donated by Dr T. Masuda (Institute for Immunology, Faculty of Medicine, Kyoto University).

SDS-PAGE. SDS-PAGE was performed by using slab gels according to the method of Laemmli.[29] The following mol wt markers (Bio-Rad, Richmond, CA) were utilized: myosin (200,000), beta-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Thromboxane (TX) B_2 assay. Platelet counts in the samples were adjusted to 3×10^5 μL. Aliquots (0.2 mL) of PRP or washed platelet suspension in HEPES-buffered saline were incubated with an agonist or saline in cuvettes at 37°C under continuous stirring on the aggregometer. After five minutes the reaction was stopped with 0.1 mmol/L indomethacin, and the cuvettes were immediately cooled on ice. Samples were centrifuged at 10,000 g for two minutes at 4°C. TXB_2 in the supernatant was assayed directly using a TXB_2 RIA kit (New England Nuclear, Boston).

Measurement of cytoplasmic free calcium ion concentration ($[Ca^{2+}]_i$). Platelet $[Ca^{2+}]_i$ was determined by using photoprotein aequorin purchased from Dr John Blinks (Mayo Clinic) by a slight modification of the method of Johnson et al.[30] Aequorin-loaded platelets were finally resuspended in HEPES-Tyrode's buffer without Ca^{2+}, and the external calcium concentration was adjusted to 1 mmol/L by the addition of $CaCl_2$ just prior to the measurement; the platelets were equilibrated at 37°C for five minutes. The elevation of platelet [Ca^{2+}]$_i$ induced by IgG was measured on a Platelet Ionized Calcium Aggregometer (Chrono-Log) with simultaneous recording of platelet aggregation. [Ca^{2+}]$_i$ was calculated according to Johnson et al.[30]

40 kDa protein phosphorylation. Washed platelets (2 × 10^9) suspended in Tris-buffered saline (25 mmol/L Tris-HCl, 145 mmol/L NaCl, pH 7.4), were incubated with 0.4 mCi of ^{32}P-orthophosphoric acid (Radiochemical Centre, Amersham, England) at room temperature for 60 minutes in a total volume of 4 mL. Platelets were then pelleted by centrifugation at 1,100 g for ten minutes and resuspended in assay buffer (25 mmol/L Tris-HCl, 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, pH 7.4) at a density of 5 × 10^5/μL. After preincubation for 10 min at 37°C, the reaction was started by the addition of IgG. Aliquots (100 μL) were transferred into tubes containing 10 μL of 11% SDS-550 mmol/L dithiothreitol[31] and were boiled for two minutes. After the samples were chilled on ice, they were subjected to 10% SDS-PAGE. The gels were stained by Coomassie brilliant blue (Nakarai Chemical, Kyoto, Japan), dried on a filter paper, and autoradiographed by exposure to Fuji X-ray film for 1 to 2 weeks. A band corresponding to 40 kDa protein was cut into a vial and counted for radioactivity in toluene scintillator (PPO 25 g, dimethyl-POPOP 1.5 g and toluene 5 L).

Absorption of IgG by FWP. Various concentrations of FWP in PBS (0 to 4 × 10^6/μL) were incubated with 2 mg/mL of the patient's or normal IgG in the presence of apyrase (Sigma) (2 mg/mL) at 37°C for one hour and then at 4°C overnight. The mixtures were centrifuged at 1,100 g for ten minutes at 4°C, and the aggregating activity of each supernatant was tested on normal PRP to assess the absorption of IgG by FWP. The change in light transmittance of normal PRP (200 μL) three minutes after the addition of 10 μL of the supernatant was used as an indicator of aggregating activity.

Elution of IgG. Washed platelets resuspended in TBS (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4) containing 0.1% bovine serum albumin (BSA, Sigma) and 1 μmol/L PGE_1 (Ono Pharmaceutical, Osaka, Japan) at a density of 1 × 10^6/μL were incubated with 1 mg/mL of the patient's purified IgG at 37°C for 30 minutes. The platelets thus sensitized were then washed three times with TBS containing 0.1% BSA and 1 μmol/L PGE_1 and finally suspended in TBS. The eluate from the sensitized platelets was prepared by lowering the pH of the medium to 3.0 by the addition of 0.1 N HCl.[32] After standing at room temperature for ten minutes, the mixture was centrifuged at 1,100 g for ten minutes. The supernatant was transferred to a tube to which a neutralizing solution (1 mol/L Tris-HCl, pH 9.0) was immediately added to a pH of 8.0. The eluate thus obtained was then applied to a protein-A Sepharose column to prepare purified IgG. After concentration on Amicon Ultrafiltration Cell, the aggregating activity of the eluate was assessed on normal PRP (200 μL).

Immunoprecipitation studies of solubilized iodolabeled platelets. Lactoperoxidase-catalyzed ^{125}I-labeling of platelets was performed as described by Nurden et al.[33] Washed platelets were resuspended at 1 × 10^6 platelets/μL in TBS. To 1 mL of each platelet suspension, 0.5 mCi ^{125}I-NaI (New England Nuclear) and 10 μL of 0.25 mmol/L lactoperoxidase (Sigma) solution in TBS were added. Finally 5 × 12 μL aliquots of 1 mmol/L H_2O_2 prepared in TBS were added at ten-second intervals. The labeled platelets were then sedimented and washed three times in TBS containing 5 mmol/L KI before being lysed in 1 mL of the lysis buffer (0.5% Nonidet P-40 [NP-40], 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.02% NaN_3, pH 7.4) containing protease inhibitors (0.2 mmol/L phenylmethylsulphonyl fluoride, 5 mmol/L N-ethylmaleimide, and 200 μg/mL leupeptin [Peptide Institute Inc, Osaka, Japan]). After centrifugation at 10,000 g for 30 minutes at 4°C, the supernatant was used for immunoprecipitation assay, which was performed essentially according to Kessler.[34] One milliliter of formalin-fixed protein A-bearing *Staphylococcus aureus* (Staph-A) in 10% suspension (Pansorbin; Calbiochem-Behring, La Jolla, CA) was washed three times at 2,500 g for ten minutes at 4°C in extraction/washing buffer (0.5% NP-40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L KI, 0.5% BSA, 0.02% NaN_3, pH 7.4). The final pellet was suspended in 1 mL of a NP-40 extract of unlabeled platelets (1 × 10^6/μL) from the same donor and incubated on ice for 15 minutes to prevent subsequent nonspecific binding of iodolabeled protein to the Staph-A. After centrifugation at 2,500 g for ten minutes at 4°C, the pellet was resuspended in the starting volume of the extraction/washing buffer (pretreated Staph-A suspension). Iodolabeled platelet extract (100 μL, 8 to 12 × 10^6 cpm) was incubated with 100 μg of purified IgG for two hours at 4°C. The pretreated Staph-A suspension (200 μL) was then added, and the mixture was incubated for 30 minutes at 4°C. After centrifugation the pellet was washed four times in the extraction/washing buffer and once in the same buffer without BSA. Finally the pellet was resuspended in 100 μL of SDS-PAGE sample buffer (2% SDS, 62.5 mmol/L Tris-HCl, 10% [wt/vol] glycerol, 0.002% bromphenol blue, pH 6.8) with or without 5% (vol/vol) 2-mercaptoethanol, and boiled for five minutes. Following centrifugation the supernatant was electrophoresed at 25 V in a 3% stacking gel and 7.5% separating gel. Whole iodolabeled platelet extract (5 μL) was also boiled in 100 μL of SDS-PAGE sample buffer for five minutes and electrophoresed in parallel with the precipitate samples. The gel was stained with Coomassie brilliant blue, dried under vacuum, and autoradiographed by exposure to Fuji X-ray films and a Fuji X-ray intensifier (Grenex-8) at −70°C for one to three days.

Protein determination. Protein measurement was done by the Lowry's method[35] using BSA as a standard.

Materials. Pepsin, papain, lactoperoxidase, creatine phosphate (CP), creatine phosphokinase (CPK), and dibutyryl cyclic adenosine monophosphate (dbc AMP) were obtained from Sigma; collagen reagent for aggregation studies from Hormon-Chemie, Munich, West Germany; arachidonic acid (AA) from Nu-Chek, Elysian, MN; platelet activating factor (PAF) and A23187 from Calbiochem-Behring; heparin from Kodama, Osaka, Japan. PGI_2 was a generous gift from Ono. Sepharose 2B was obtained from Pharmacia Fine Chemicals. All other reagents were the same as those described previously[23] or were obtained from Nakarai, Kyoto, Japan.

RESULTS

Characteristics of the patient's platelets. Platelet aggregation in the patient's PRP was normal in response to ADP, epinephrine, AA, ristocetin, A23187, and PAF (Fig 1). Thrombin-induced aggregation was also normal in washed platelet suspension (data not shown). In contrast, collagen (up to 10 μg/mL) induced neither shape change nor aggregation in PRP (Fig 1) or in washed platelet suspension (data not shown). The potentiating effect of epinephrine on collagen-induced aggregation was absent, whereas that on ADP- and AA-induced aggregation was observed, as is normal (data not shown). ATP release of PRP, as determined by Lumiaggregometer, was normal in response to these stimuli other than collagen, which induced no ATP release (data not shown). These results were consistently found over a period of two years. Adhesion of the patient's platelets to collagen fibrils was 13.7%, 27.5%, and 50.2% at 15, 30, and 60 μg/mL of collagen, respectively. These values were lower than the normal ranges at all collagen concentrations studied (normal

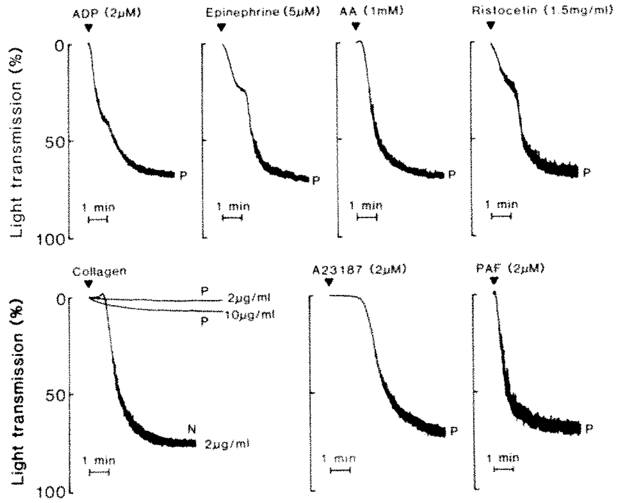

Fig 1. **Aggregation tracings of the patient's and control PRP. Aggregating agents whose final concentrations are shown in parentheses or at the end of aggregation curves were added as indicated (▼). Aggregation patterns of control PRP in response to each agonist other than collagen are not shown because they are similar to those of the patient's PRP. P, patient's PRP; N, control PRP.**

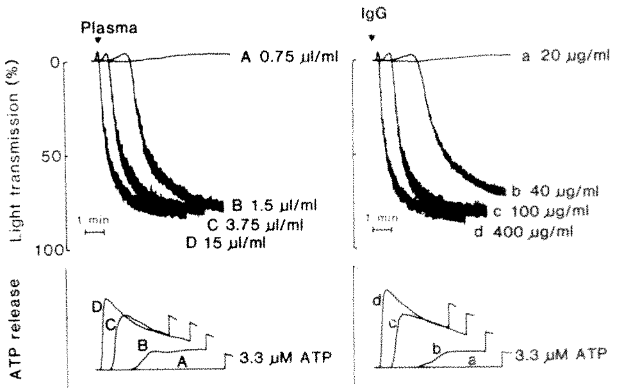

Fig 2. **Dose dependency of aggregation and ATP release of normal platelets induced by the patient's PPP (plasma) and IgG. Plasma or IgG of the patient was added to normal PRP as indicated (▼) at various concentrations that were shown at the end of each aggregation curve.**

ranges [M ± 2 SD, n = 13]: 28.5% ± 12.4%, 41.0% ± 9.0%, and 59.2% ± 6.2%, respectively). Thus in contrast to defective collagen-induced aggregation, some adhesion to collagen was always observed.

Platelet ATP and ADP contents were 6.99 and 2.42 μmol/10^{11} platelets, respectively, and the ratio of ATP to ADP was 2.88. These values were all within normal ranges (6.69 ± 1.09, 2.98 ± 0.99, and 2.47 ± 0.93, respectively; n = 17).

TXB_2 was normally produced in response to thrombin (0.5 U/mL), but there was no significant TXB_2 synthesis on stimulation with collagen (2 μg/mL) in the patient's platelets (Table 1).

Platelet membrane GP profiles (GP Ia, Ib, IIa, IIb, IIIa, and IIIb) were normal in one-dimensional SDS-PAGE (7.5% acrylamide gel) as determined by autoradiographs of ^{125}I-labeled platelets (vide infra).

Aggregation and the release reaction of normal platelets induced by the patient's plasma and IgG. The patient's plasma induced irreversible aggregation and ATP release in normal PRP in a concentration-dependent manner (Fig 2). The aggregation response was associated with a transient decrease in light transmission and a lag period, during which ATP release was not observed (Fig 2, lower panels). The minimal concentration of the patient's IgG required for aggregation of platelets obtained from 22 normal subjects remained within a range of 20 to 40 μg/mL. In the absence of stirring, the patient's plasma and IgG induced no aggregation, but ATP release was observed on Lumiaggregometer (data not shown). Washed platelets were also aggregated by the patient's plasma and IgG (data not shown). The patient's plasma and IgG did not induce agglutination of normal FWP in the aggregometer. LDH release by the patient's IgG (100 μg/mL), collagen (5 μg/mL), and saline were 3.4%, 3.3%, and 3.0%, respectively (mean of three experiments). Purified $F(ab')_2$ fragments (50 μg/mL) of the patient's IgG induced platelet aggregation and ATP release with patterns that were similar to those induced by intact IgG (data not shown). Fc fragments (up to 500 μg/mL) were not effective (data not shown).

The patient's Fab fragment did not induce aggregation up to a concentration of 1 mg/mL in eight of ten normal PRP, two of which were aggregated, however, by a concentration of 200 μg/mL and 500 μg/mL, respectively. Between these two groups of normal PRP, defined by the presence or absence of platelet aggregation induced by the Fab fragment, there was no obvious difference in the patterns of aggregation induced by collagen and by the patient's IgG as well as by other agonists, including ADP, epinephrine, and AA (data not shown). When normal PRP, which was not aggregated by the patient's Fab fragment was used, pre-incubation of the PRP with the Fab fragment for five minutes at 37°C inhibited aggregation induced by the patient's IgG and by collagen in a concentration-dependent manner (Fig 3). This inhibitory effect of the Fab fragment was complete in all of four normal PRP studied and reached maximum after pre-incubation for five minutes (data not shown). The Fab fragment had no inhibitory effect on aggregation induced by ADP, epinephrine, AA, and ristocetin (data not shown).

Effects of platelet inhibitors on platelet aggregation induced by the patient's IgG. The effects of platelet inhib-

Table 1. **TXB_2 Production by Platelets**

Platelets	Agonist	TXB_2 (ng/10^8 platelets)	
		Patient	Normal*
PRP	Collagen (2 μg/mL)	0.19	38 ± 6 (5)
	Saline	0.25	0.27 ± 0.07 (5)
Washed platelets	Thrombin (0.5 U/mL)	208	205 ± 20 (7)
	Saline	1.2	1.8 ± 0.3 (7)

*Mean ± SD; (n).

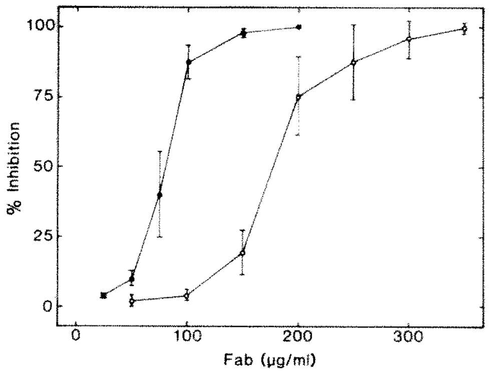

Fig 3. Inhibitory effects of the patient's Fab fragments on platelet aggregation induced by the patient's IgG and by collagen. After the five-minute pre-incubation of normal PRP with various concentrations, as indicated, of the patient's Fab fragments at 37°C, aggregation of the normal PRP was initiated by the stimulation with the patient's IgG (100 µg/mL) or collagen (5 µg/mL). Percent inhibition of aggregation was calculated by the change in light transmittance seven minutes after the addition of patient's IgG (○) and by the maximal change in light transmittance induced by collagen (●). Each value represents M ± SD from four experiments.

iting agents on platelet aggregation induced by the patient's IgG were examined (Table 2). PGI$_2$ and dbc AMP (inhibitors by way of cyclic AMP accumulation) and diltiazem (a calcium blocker) completely inhibited this aggregation. A calcium chelator, EDTA, also inhibited aggregation but not completely, even when the concentration of EDTA was increased up to 10 mmol/L. Metabolic inhibitors, iodoacetate plus antimycin A, completely inhibited aggregation, whereas either of them alone was partially inhibitory. ADP scavengers, apyrase and CP/CPK, as well as cyclooxygenase inhibitors, aspirin and indomethacin, only slightly affected aggregation by the patient's IgG. A thrombin inhibitor, heparin, had no effect on platelet aggregation induced by the patient's IgG.

TXB$_2$ synthesis, [Ca^{2+}]$_i$ elevation, and 40 kDa protein phosphorylation in normal platelets induced by the patient's IgG. TXB$_2$ synthesis by normal platelets was induced by the patient's IgG in a concentration-dependent manner (0.4, 14, 64, 104, and 181 ng TXB$_2$/10^8 platelets by 20, 40, 100, 200, and 400 µg/mL of the patient's IgG, respectively; mean of two experiments). Figure 4 shows simultaneous tracings of [Ca^{2+}]$_i$ elevation and aggregation of normal platelets induced by the patient's and control IgG. Stimulation with the patient's IgG elevated [Ca^{2+}]$_i$ with the same lag period as in platelet aggregation (Fig 2). The extent of [Ca^{2+}]$_i$ elevation was comparable with that caused by stimulation with thrombin (0.5 U/mL) and collagen (5 µg/mL), ie, 10 and 5.6 µmol/L, respectively. Activation of platelets by the patient's IgG was accompanied by phosphorylation of a 40 kDa protein, which reached a peak within two minutes after stimulation (Fig 5). Control IgG induced neither [Ca^{2+}]$_i$ elevation nor protein phosphorylation.

Absorption and elution of IgG. To investigate whether the patient's IgG specifically reacted with platelet membrane antigen, we performed the following experiments. Incubating the patient's IgG with increasing numbers of FWP decreased the aggregating activity of the supernatant progressively, and no residual activity was detectable in the experiment using 4 × 10^6 FWP/µL. At a protein concentration of 8 µg/mL, the eluate prepared from platelets incubated with the patient's IgG induced platelet aggregation that was comparable to aggregation induced by 40 µg/mL of the patient's original IgG.

Immunoprecipitation of radiolabeled platelet antigens with the patient's IgG. Electrophoresis of radiolabeled platelet proteins and antigens gave patterns as shown in Fig 6. The electrophoresis of whole radiolabeled platelet extract (A and C lanes) showed that platelet GP profiles (GP Ia, Ib, IIa, IIb, IIIa, IIIb)[33] of the patient's platelets (C lanes) were similar to those of normal platelets (A lanes). From the normal platelet extract, the patient's IgG immunoprecipi-

Table 2. Effects of Platelet Inhibitors on Platelet Aggregation Induced by the Patient's IgG

Inhibitor	Concentration	% Inhibition
PGI$_2$	10 nmol/L	100 ± 0*
dbc AMP	4 mmol/L	100 ± 0
EDTA	5 mmol/L	74 ± 6
Diltiazem	1 mmol/L	100 ± 0
Aspirin	1 mmol/L	3 ± 1
Indomethacin	100 µmol/L	1 ± 1
Apyrase	2 mg/mL	7 ± 6
CP/CPK	3 mmol/L/15 U/mL	3 ± 2
Iodoacetate	0.5 mmol/L	59 ± 8
Antimycin A	2 µmol/L	2 ± 1
Indoacetate/Antimycin A	0.5 mmol/L/2 µmol/L	100 ± 0
Heparin	2 U/mL	0

*Mean ± SD; n = 3.

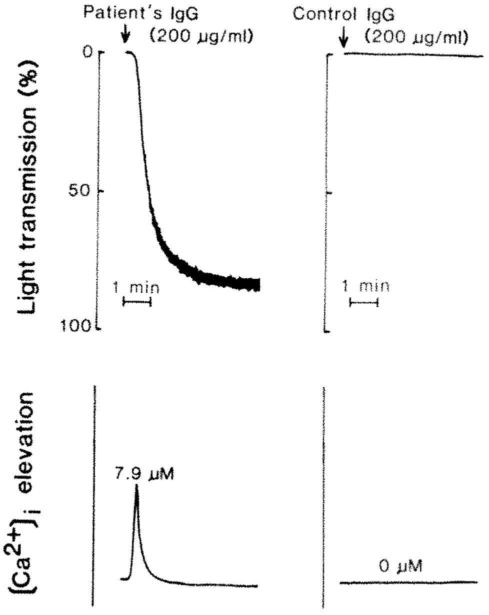

Fig 4. Simultaneous measurement of [Ca^{2+}]$_i$ elevation and aggregation of aequorin-loaded normal platelets induced by the patient's and control IgG. IgG (200 µg/mL) prepared from the patient and normal subjects was added to aequorin-loaded platelets from normal subjects as indicated by the arrow.

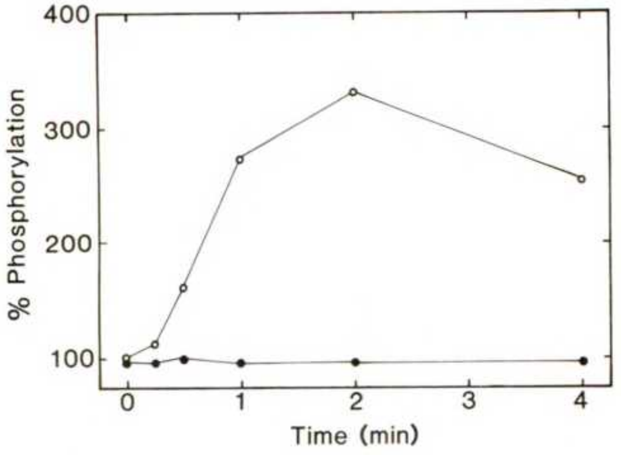

Fig 5. Time course of 40 kDa protein phosphorylation in normal platelets stimulated by IgG. Normal platelets ($5 \times 10^5/\mu L$) prelabeled with ^{32}P were exposed to the patient's (○) or normal (●) IgG (100 $\mu g/mL$), and aliquots (100 μL) were transferred to the stop solution after varying periods of time as indicated, followed by SDS-PAGE, autoradiography, and the determination of radioactivity as described in Materials and Methods section. Each value represents mean from three experiments.

tated five polypeptide bands in both reduced and unreduced states (B lanes). The protein with an apparent mol wt of 62 kDa (reduced state) and of 57 kDa (unreduced state) was by far the most prominent band, suggesting that this protein played a major role in the interaction between the patient's IgG and platelets. Two of the minor polypeptides were identified as GPIIb and GPIIIa from their migration patterns in the reduced and unreduced state.[33,36] Two other proteins had mol wt of more than 200 kDa in the reduced state, and one of them had a mol wt of 190 kDa in the unreduced state. In contrast, no significant immunoprecipitate was detected when an extract of the patient's platelets reacted with the patient's IgG (D lanes). Control IgG prepared from normal plasma precipitated no significant protein from any platelet extracts (data not shown).

DISCUSSION

In the present study we described a patient with steroid-responsive ITP associated with Graves' disease. In spite of adequate platelet counts ($>140{,}000/\mu L$) after steroid therapy, the patient showed a mild bleeding tendency due to platelet dysfunction, which seemed to be derived from the action of antibody. The predominant lesion in the qualitative platelet disorders observed by others in association with antiplatelet antibodies resembled that of storage pool disease; the impairment of platelet functions was detected in response to several agonists,[5-10] and the patients' platelets had decreased adenine nucleotide contents.[6,9] In contrast to those patients, our patient's platelets showed both normal adenine nucleotide contents and a specific defect only in response to collagen. Whereas other agonists induced normal platelet responses, collagen induced none of the usual platelet responses, including shape change, aggregation, ATP release, and TXB_2 synthesis. These results suggested that only collagen failed to trigger the intracellular events that are normally produced or transmitted across the plasma membrane, and that the lesion in the patient's platelets may exist in a membrane component related to a collagen binding site.

Rao et al[37] reported that a patient with autoimmune thrombocytopenia showed an impairment of only collagen-induced platelet responses, including aggregation and adhesion. However, our patient is clearly distinct from this case since their patient's plasma inhibited collagen-induced aggregation of normal platelets and since collagen-induced aggregation of their patient's platelets was restored by combined stimulation with epinephrine. Clancy et al[5] reported the existence of platelet-inhibiting activity in globulin fraction from ITP patients associated with a qualitative platelet disorder. In contrast to their report, however, our patient's plasma caused aggregation and ATP release in normal PRP, indicating that there was no circulating inhibitor but a platelet-activating factor in the patient's plasma. This factor was found in the IgG fraction purified by protein-A Sepharose. Aggregation and ATP release induced

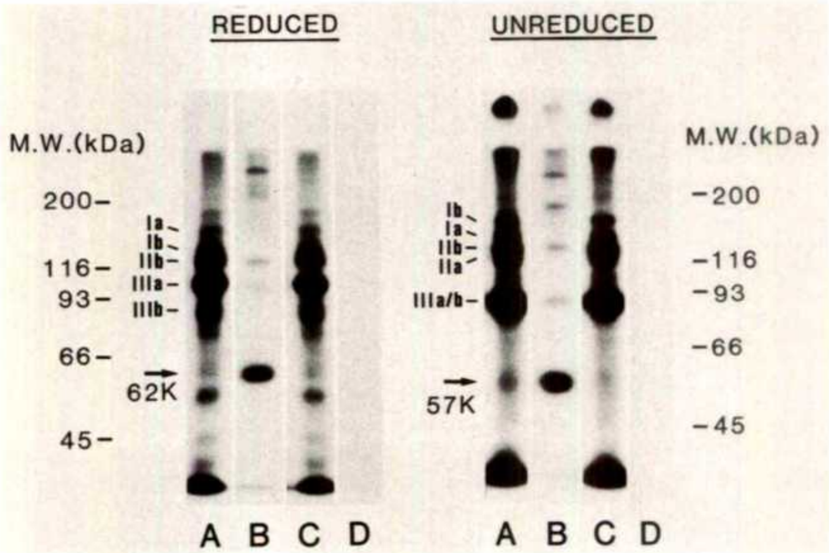

Fig 6. Autoradiographic patterns of NP-40-solubilized radiolabeled membrane proteins from normal platelets (A and B lanes) and the patient's platelets (C and D lanes). A and C lanes, whole NP-40 extract (5 μL); B and D lanes, precipitates from the patient's IgG-treated NP-40 extracts. Note that the patient's IgG immunoprecipitated five apparent proteins (B lanes) in both reduced and unreduced states from the normal platelet extract but that no such proteins were precipitated from the patient's own platelet extracts (D lanes). No precipitate was observed in normal IgG-treated normal and the patient's NP-40 extracts (data not shown).

by this factor were not attributable to cell lysis because negligible LDH activity was released by the patient's IgG. Furthermore, the responses must be independent of complement since the reaction was also observed in a complement-free system containing washed platelets and purified IgG. It has been reported that some MoAbs induced platelet activation in the absence of complement through reaction with specific antigens on the platelet membrane.[38-40]

Patient's IgG caused TXB_2 synthesis, $[Ca^{2+}]_i$ elevation, and 40 kDa protein phosphorylation in normal platelets, suggesting that platelet activation was brought about by the stimulus-response coupling pathway as seen in other physiologic agonists, including collagen and thrombin. Some antiplatelet antibodies agglutinate platelets by a passive antigen-antibody reaction (formation of an antibody-platelet lattice) independently of calcium.[40] This possibility can be ruled out for the patient's IgG because (1) the patient's IgG failed to agglutinate FWP, which could still absorb it, and (2) the patient's IgG-induced platelet aggregation was inhibited by an increase of intracellular cyclic AMP and required an intracellular Ca^{2+} flux and metabolic energy, although it was not dependent on released ADP, TXA_2 synthesis, or thrombin participation. The activity could be absorbed by and eluted from platelets, suggesting that the IgG was an antibody to a specific antigen on the platelet membrane. Furthermore, the binding site of the antibody was distinct from the Fc receptor on platelets, which mediates platelet activation by IgG aggregates[41] or immune complexes[42] because $F(ab')_2$ fragments prepared from the patient's IgG induced platelet aggregation as well as ATP release and because Fab fragments from the patient's IgG completely abolished the aggregation of normal platelets induced by the patient's IgG. It is possible that the action of the patient's IgG may be directed at or near a putative collagen receptor since only collagen-induced aggregation was inhibited by Fab fragments of the patient's IgG. Thus the specific binding of the patient's IgG to the specific antigen on the platelet could induce a conformational change of the platelet membrane and/or a direct transmembrane signal, thereby allowing platelet activation.

The binding site of the patient's IgG was examined by immunoprecipitation of radiolabeled, detergent-solubilized platelet membrane proteins and SDS-PAGE. From normal platelet extract, the patient's IgG precipitated five polypeptides, of which a polypeptide of 62 kDa (reduced state) (57 kDa [unreduced state]) was the main protein, as judged from the intensity of radioactivity. There are two potential explanations for the precipitation of these multiple proteins: (1) the presence of antibodies against several different antigens; (2) the coprecipitation of a multicomponent complex by an antibody directed against only one of the components. The 62 kDa protein, the most prominent precipitate seen in our study, could be similar to the 65 kDa protein, which was isolated by using chick skin collagen immobilized on Sepharose beads,[16] or to the 61 kDa protein, which has recently been reported to bind insoluble collagen.[20] However, more direct experimental evidence is needed to substantiate the identity of these proteins. Nieuwenhuis et al[43] reported a patient with platelets that were totally unresponsive to collagen and did not express surface GPIa, and they suggested that GPIa had collagen receptor activity. Our data, however, do not support this notion because our patient's platelets had normal GPIa expression in spite of defective collagen-induced aggregation.

Our immunoprecipitation studies could not demonstrate any significant reaction of the patient's IgG with her own platelet extract, although the electrophoresis of whole platelet extracts showed that the patient's platelets were iodolabeled in the same way as normal platelets. Our experimental results imply that no additional antigen-antibody reaction could be induced in vitro using the patient's platelets and that the patient's platelets may not be activated by her own IgG. Furthermore the absence of the antigens, including the putative collagen receptor, could explain the defect of the patient's platelet aggregation only in response to collagen. However, the meaning of the absence of such antigens on the patient's platelet membrane is unknown. One possibility is that these antigens were lost or denatured, leading to destruction of their antibody-specific epitopes. This could occur during the peripheral circulation of the platelets and/or during the maturation of megakaryocytes because antiplatelet antibodies also react with marrow megakaryocytes.[1] In a well-defined autoimmune disorder, myasthenia gravis, the acetylcholine receptor on the postsynaptic membrane is denatured and decreased by the action of antiacetylcholine receptor autoantibody through complement-mediated destruction[44] or endocytosis.[45] By analogy, the antigen possibly responsible for collagen-binding on the platelet membrane may be denatured in the patient's platelets, preventing their response to collagen.

In our patient platelet adhesion to collagen fibrils was detectable, although it was significantly decreased compared with platelet adhesion in normal subjects. These results suggest that platelet membrane sites responsible for aggregation and adhesion are different, and the results support the concept of Kotite et al[18] and Pintigny et al[46] that collagen-induced platelet activation consists of a recognition (adhesion) step and an intermediary step (effect of collagen recognition) leading to postreceptor reactions, including the release of granular constituents and fibrinogen binding to platelets. Pintigny et al[46] demonstrated that a synthetic octapeptide derived from type III collagen inhibited collagen-induced platelet aggregation without affecting platelet adhesion to collagen, indicating that the octapeptide reacted with the site of the intermediary step but not with the recognition site.[46] Similarly, the defect on our patient's platelets may reside in the site of the intermediary step.

In conclusion, we found a novel antiplatelet antibody in an ITP patient associated with defective collagen-induced platelet aggregation. The antibody induced platelet aggregation and ATP release in PRP as well as in a washed platelet suspension prepared from normal subjects. This antibody showed specificity against antigens, including one that seemed to be related to collagen-binding sites on normal platelets. Thus these studies may serve as a useful tool for further investigation of the platelet membrane constituents involved in collagen-induced platelet activation.

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Liver Transplantation in Hemophilia A

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Four patients with hemophilia A have undergone liver transplantation in our institution, three successfully. The first was a 21-year-old man with chronic active hepatitis (CAH) in whom the effects of previous abdominal operations prevented the satisfactory technical insertion of the new liver. He died intraoperatively. The second patient was a 15-year-old boy with CAH who began to synthesize factor VIII coagulant activity (F VIII:C) within 18 hours of successful liver transplantation and has continued to do so for almost 2 years (F VIII:C range 0.89 to 3.20 U/mL). The first 2 months of his postoperative course were complicated by infections, but since that time he has done well and has returned to school. The third patient was a 48-year-old man with portal fibrosis and severe ascites. He synthesized F VIII:C (range 0.96 to 1.50 U/mL) within six hours after reestablishment of circulation through the new liver. His postoperative course was complicated by numerous infections, and he died with sepsis and an acquired immunodeficiency-like syndrome 4 months after transplantation. The fourth patient was a 47-year-old mild hemophiliac with CAH who produced adequate factor VIII:C levels following transplantation (range 0.79 to 2.80 U/mL). These patients demonstrate that liver transplantation in hemophiliacs with end-stage liver disease may be lifesaving and results in correction of the F VIII:C deficiency and associated hemorrhagic tendency.

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WHILE the site of production of human factor VIII coagulant activity (F VIII:C) has been unclear, data generated during the past two decades have consistently implicated the liver as the primary site of synthesis. This was accomplished utilizing liver perfusion techniques[1-3] and orthotopic hepatic transplantation into hemophiliac dogs.[4] However, others have suggested that the spleen[5-7] or lung[8] were important as well. Recent immunohistologic advances have localized F VIII:C primarily to the liver[9,10] and in particular to hepatic sinusoidal cells.[10] Whether these cells are the site of synthesis or are merely serving a storage function is not known. By recombinant DNA techniques,[11-14] F VIII:C messenger RNA has been demonstrated in the hepatocyte[14] as well as the kidney, although the latter was not identified as a site of origin.[15] The first successful human orthotopic liver transplantation in a F VIII-deficient hemophiliac was recently reported in a letter from this laboratory.[16] The new liver reversed the F VIII:C defect. The purpose of this report is to describe in detail the orthotopic liver transplantation in four F VIII:C-deficient hemophiliacs. All patients were advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent. In the three who survived the surgery, F VIII:C levels normalized, and their bleeding problems resolved.

CASE PRESENTATIONS

Patient 1 was a 21-year-old white, moderately F VIII:C-deficient hemophiliac who had received multiple infusions of F VIII concentrate in the past and had developed severe hepatic dysfunction. When first tested his blood showed antibody to hepatitis B surface antigen (anti-HBs), although no definite history of acute hepatitis could be obtained. A biopsy diagnosis of CAH was made in 1973; variceal bleeding became evident in 1976; and he underwent a splenectomy and a side-to-side portacaval shunt. In 1977 he had an appendectomy for a perforated appendix and in 1979 a cholecystectomy for cholelithiasis. During the last 6 years of his life he had frequent episodes of hepatic encephalopathy in addition to hemophilia-associated symptoms such as intramuscular (IM) hematomata and hemarthroses. Preoperative laboratory tests are shown in Tables 1 through 3. After a thorough evaluation the patient was accepted for transplantation; a donor liver became available approximately 6 weeks later; and the procedure was performed on May 13, 1982. Intraoperatively he utilized 15,950 units of F VIII concentrate. The technical aspects of the transplant were greatly complicated by foreshortened recipient vessels, a result of previous operations, which made the construction of vascular anastomoses difficult. Venovenous bypass during the anhepatic phase, which decompresses the portal circulation, was not being used at that time. When the portal and caval venous circulations were occluded, the intestines became edematous, and uncontrollable bleeding ensued. A total of 55 units of RBCs and 61 units of fresh frozen plasma were transfused, but the patient died on the operating table.

Patient 2 is a 16-year-old white, severely F VIII:C-deficient hemophiliac who had been treated with F VIII concentrate since early childhood. During the first year of life he had severe hepatitis B, which he transmitted to both parents. Subsequently hepatitis B surface antigen (HBsAg), anticore antibody (anti-HBc), and antibody to hepatitis B e antigen (anti-HBe) persisted. Additional laboratory tests are shown in Tables 1 through 3. In February 1984 he developed liver enzyme elevations that may have represented non-A, non-B (NANB) hepatitis or possibly a concurrent delta agent infection. In October 1984 he developed severe thrombocytopenia, which resolved following splenectomy. A simultaneous liver biopsy showed CAH. Postoperatively he developed a pancreatogastrocutaneous fistula that persisted for several months. Liver transplantation and repair of the fistula were performed on March 5, 1985. There was a small accumulation of ascites, but little difficulty was encountered during the transplant procedure, which required only four units of RBCs. The fistula repair included excision of numerous sinus tracts and required eight units of RBCs. During the operative and immediate postoperative period he was given 14,160 units of heat-treated F VIII concentrate. Eighteen hours postoperatively F VIII infusions were discontinued, and the F VIII:C levels

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Table 1. Coagulation Studies

Test	Normal Range	Patient 1 3/5/85* 3/8/85†	Patient 2 3/5/85;* 2/14/85; 3/25/85†		Patient 3 10/19/85;* 10/9/85; 10/28/85†		Patient 4 6/30/86;* 6/17/86; 7/9/86†	
Prothrombin time (s)	10-13	16.6	10.9	11.1	14.4	11.3	14.0	12.6
Activated partial thromboplastin time (s)	24-34	107.0	39.0	31.9	53.9	29.8	60.7	46.8
Thrombin time (s)	13-18	40.4	30.5	13.0	27.5	17.3	20.7	22.0
Reptilase time (s)	13-18	50.4	35.1	—	33.6	—	29.9	—
Euglobulin lysis time (min)	120 or >	90	240	240	15	150	90	210
Ethanol gel (0-4)	0	0	2	1	0	0	0	—
Protamine gel (0-4)	0	0	2	0	0	1	0	—
Wellcotest (μg/mL)	0	0	>40	>10<40	>40	0	0	—
Staph clumping titer	1-4	0	0	8	128	0	0	0
Platelet count ($10^3/\mu$L)	150-450	73	344	414	130	183	50	130

*Date of surgery.

†Dates of tests.

thereafter remained normal or supranormal. On the ninth postoperative day he underwent revision of the bile duct reconstruction (initially performed during the transplantation), and on the 28th postoperative day a subphrenic abscess was drained. Both procedures were performed without F VIII replacement therapy, and no excess bleeding was encountered. Pre-operative and postoperative coagulation findings are shown in Tables 1 and 2.

Six weeks postoperatively he was discharged, and since that time he has maintained F VIII:C levels of 0.89 to 3.20 U/mL. He was given 20 vials (100 mL) of hepatitis B immune globulin (HBIG) intraoperatively and postoperatively. His HBsAg has remained positive except for several weeks (beginning about five weeks postoperatively) when it was negative and anti-Hbs appeared. Since then he has remained positive for HbsAg, anti-Hbs, and the antibody to delta agent. He returned to temporary employment in the summer of 1985 and returned to school in the fall of 1985.

Patient 3 was a moderately obese, 48-year-old white, severely F VIII:C-deficient hemophiliac on home treatment with F VIII concentrate as needed since 1973. Since that time his bilirubin, SGPT (ALT), and SGOT (AST) had been intermittently elevated and his anti-HBs had been positive. The patient had a strong history of alcohol abuse in the past but discontinued its use in 1974. Liver biopsy in 1976 showed portal fibrosis with fatty changes and early nodule formation. His liver disease was moderately stable until July of 1985 when there was a marked increase in girth due to development of massive ascites. The spleen was enlarged; the gallbladder was contracted and contained gallstones. Subsequent progressive jaundice, malaise, accumulation of ascites, and poor response to diuretics heralded further clinical deterioration. Pre-operative and postoperative laboratory findings are shown in Tables 1 through 3. The patient was evaluated favorably for liver transplantation, and the procedure was performed on October 19, 1985. Twelve liters of ascitic fluid were removed. He received 17,290 units of heat-treated F VIII concentrate and ten units of RBCs intraoperatively. Postoperatively the patient received no F VIII concentrate, and his factor VIII:C level ranged from 0.96 to 1.50 U/mL. An episode of postoperative bleeding of unknown cause necessitated a repeat laparotomy. No excessive operative bleeding was encountered, and no F VIII concentrate was required. The patient suffered a mild rejection crisis and was successfully treated with OKT3 (Ortho Pharmaceuticals, Raritan, NJ) monoclonal antibody (MoAb) therapy. The rejection crisis was followed by pneumocystis carinii pneu-

Table 2. Coagulation Factor Assays (Pre- and Post-Liver Transplant)

Factor		Patient 1	Patient 2		Patient 3		Patient 4	
		Pre	Pre	Post	Pre	Post	Pre	Post
		3/03/82	8/27/84	3/19/85	3/11/81	11/1/85	6/17/86	7/15/86
VIII:C	U/mL*	0.02†	0.01†	1.75†	0.01†	1.15†	0.10†	2.80†
vwF		10.0	6.24	3.68	1.06	6.24	2.32	3.36
RCF		4.35	3.75	4.64	0.97	4.20	3.40	2.76
		5/13/82	2/14/85	3/25/85	10/09/85	10/28/85	6/17/36	7/9/86
I	mg/dL	180	375	420	240	270	160	440
II	U/mL*	0.3	0.78	0.90	0.48	0.70	0.46	0.80
V		0.57	0.80	0.96	0.36	1.25	0.22	0.44
VII		0.18	0.68	0.66	0.23	0.60	0.35	0.58
VIII:C		0.11‡	1.30‡	1.20†	0.33‡	1.40†	0.10†	1.10†
IX		0.11	0.95	1.00	0.26	0.86	0.48	0.90
X		0.49	0.80	1.05	0.68	0.58	0.56	1.05
XI		0.15	0.39	0.63	0.24	0.52	0.19	0.55
XII		0.36	0.91	0.66	0.67	0.78	0.43	0.63

*Normal range, 0.5 to 1.5 U/mL.

†Not treated with F VIII.

‡Treated with F VIII.

Table 3. Preoperative Hepatitis Serologies, Liver Function Tests, and Anti-HIV Results

Test	Patient 1	Patient 2	Patient 3	Patient 4
HBsAg	0	+	0	0
HBeAg	ND	–	ND	ND
Anti-HBe	ND	+	ND	ND
Anti-delta	ND	+	ND	ND
Anti-HBs	+	0	+	+
Anti-HBc	+	+	+	+
Bilirubin (mg%):				
Total	5.0	3.5	25.1	3.0
Direct	3.0	1.4	12.7	0.9
SGOT (AST) (IU)	202	313	120	48
SGPT (ALT) (IU)	80	79	62	26
Anti-HIV ELISA	+	+	+	–
Western blot	+	+	+	ND

ND, not done.

monia, which resolved with trimethoprim-sulfamethoxazole therapy. Subsequently a cytomegalovirus infection resolved without treatment. Four months postoperatively he developed *Escherichia coli* and pseudomonas sepsis, to which he succumbed despite parenteral antibiotics and intensive care.

Patient 4 is a 47-year-old mildly F VIII:C-deficient hemophiliac who had a F VIII:C level of 0.10 U/mL and a history of CAH secondary to NANB hepatitis accompanied by episodes of variceal bleeding, encephalopathy, and cholecystitis. Liver transplantation was performed on June 30, 1986 with 17,120 units of heat-treated F VIII concentrate and eight units of RBCs transfused intraoperatively. Preoperative and postoperative coagulation findings are shown in Tables 1 and 2. No factor VIII replacement was given postoperatively, and the new liver began synthesizing F VIII:C within four hours after the procedure. His postoperative course was complicated by a mild rejection episode, which was also treated successfully with OKT3 MoAb, and the patient was discharged approximately 8 weeks postoperatively after maintaining F VIII:C levels between 0.79 and 2.80 U/mL.

DISCUSSION

Successful liver transplantation in the hemophiliacs presented here not only reversed the hepatic failure but also corrected the coagulation defect of hemophilia A. These results demonstrate that hemophilia should not be a contraindication for the procedure and that resolution of the hemophilia, except for genetic transmissibility, should be expected. Therefore consideration of this procedure as a feasible, albeit difficult option should be given to the small but significant number of hemophiliacs who suffer from end-stage liver disease. The fatal outcome of Patient 1 suggests that scarring and fibrous changes after abdominal surgical procedures, especially shunting procedures and splenectomy, may make liver transplantation difficult or impossible and should be avoided. Whether or not the presence of human immunodeficiency virus (HIV) antibodies in a transplant candidate will alter the selection process remains to be determined. Three of these four patients were HIV antibody positive, and one of the three suffered from opportunistic infections typical of acquired immunodeficiency syndrome (AIDS) or the immunosuppressed post-transplantation state.[17] The diagnostic criteria of the Centers for Disease Control (CDC), however, exclude the diagnosis of AIDS in patients receiving immunosuppressive agents. At this point exclusion of anti-HIV-positive hemophiliacs for liver transplant consideration seems to be premature.

Although liver transplantation with its attendant risks and requirements for lifelong immunosuppression cannot be recommended as a treatment for hemophilia per se, it clearly may avert imminent death in hemophiliacs with end-stage liver disease and also appears to cure the coagulation defect. Future prospects for the availability of recombinant F VIII:C products may decrease the present high risk of transmissible liver disease[18] and eventually obviate the need for liver transplantation in hemophilia.

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Ultrastructural Analysis of Human Natural Killer Cell Activation

By Daniela Zarcone, Edgar F. Prasthofer, Fabio Malavasi, Vito Pistoia, Albert F. LoBuglio, and Carlo E. Grossi

In this study we describe characteristic ultrastructural changes of CD3⁻ large granular lymphocytes (LGL), ie, natural killer (NK) cells, following stimulation with recombinant (r) interleukin 2 (IL 2) or r-γ interferon (r-γ IFN) and after interaction with K562 target cells (TC) or Sepharose-bound anti-Fc γ receptor (FcR) monoclonal antibody (MoAb). When compared to resting cells the cytolytic activity of r-IL 2- and r-γ IFN-stimulated cells against K562 TC was enhanced. The r-IL 2-stimulated LGL were larger and consistently displayed the shape and cytoskeletal rearrangement characteristic of activated cells. The Golgi apparatus was expanded, and the number of electron-dense granules and vesicles was increased. The ultrastructural changes in r-γ IFN-stimulated LGL were markedly different from those observed following r-IL 2 activation. Cells did not exhibit changes in size, shape, cytoskeletal organization, or in the structure of the Golgi apparatus. However, r-γ IFN-stimulated cells exhibited distinctive changes in the structure and content of electron-dense granules with deaggregation of the matrix and parallel tubular arrays (PTAs). Within organelles apparently derived from the electron-dense granules, vesicular and tubular structures were noted that may be the morphological equivalent of cytotoxic factors produced by cytolytic effector cells. These ultrastructural observations indicate that r-IL 2 and r-γ IFN enhance the lytic ability of NK cells by acting on distinct cell machineries. The cytolytic ability was decreased when LGL were pretreated with K562 TC or immobilized anti-FcR antibody. In both experimental conditions cells displayed ultrastructural features indicating activation as well as loss of cytoplasmic granules and other Golgi-derived organelles. Stimulation of r-γ IFN- or r-IL 2-activated LGL with K562 TC or Sepharose-bound anti-FcR antibody decreased their cytolytic ability, with cells depleted of granules at the ultrastructural level. Intracytoplasmic fusion of granules and a massive release of the granule content were found in r-IL 2-stimulated cells, reminiscent of the mechanism of basophil degranulation. These observations suggest that multiple activation signals involving distinct surface membrane molecules induce release of cytolytic factors by both resting and activated NK cells.

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NATURAL killer (NK) cells are a subset of peripheral blood lymphocytes with distinctive morphological, phenotypic, and functional characteristics. NK cells display the structural features of large granular lymphocytes (LGL)[1-4] and the ability to lyse a variety of neoplastic and non-neoplastic cell targets, including virus-infected cells and hematopoietic cell precursors.[5-7] The phenotype of NK cells is best defined by the expression of receptors for the Fc portion of IgG (FcR, CD16) in the absence of the T cell receptor-associated complex, CD3.[6,8,9] FcR is the membrane ligand that allows recognition of IgG antibody-coated target cells (TC) for antibody-dependent cell-mediated cytotoxicity (ADCC), a cytolytic function exerted by cells that are virtually indistinguishable from NK cells.[3]

TC lysis is the final step in a cascade of events initiated by the formation of conjugates between NK cells and TC.[7,10-12] Following interaction with the appropriate TC, NK cells show increased motility, redistribution of Golgi apparatus, microtubule organizing center, and other cytoskeletal structures.[13-17] These events are commonly referred to as NK cell activation or NK cell programming for TC lysis. As a consequence of activation, NK cells release the matrix of their granules, which is cytolytic for TC. The nature of the signals that determine NK/TC conjugate formation as well as programming of the NK cell for TC killing are presently unknown. However, in accordance with that observed for T or B lymphocytes, it is feasible that activation signals are delivered to NK cells via surface membrane molecules, and it appears that the FcR may be one such molecule.[18,19]

Another facet of the NK cell-activation process is demonstrated by enhancement of cytolytic efficiency by γ interferon (γ IFN) or interleukin 2 (IL 2).[20,21] Both lymphokines are capable of boosting NK cell-mediated cytolysis independently of cell proliferation.[22] Furthermore, following IL 2 activation blood mononuclear cells acquire the ability of lysing TC that are refractory to the lytic effect of resting NK cells (eg, fresh tumor cells or tumor cell lines), a phenomenon known as lymphokine-activated killing (LAK).[23,24] Whether γ IFN and IL 2 exert their effect on NK cells or they recruit other types of cytolytic cell precursors is still a matter of debate.[25,26]

In this study we describe changes in the ultrastructural features of LGL following interaction with the NK-susceptible K562 cell line or with Sepharose-bound anti-FcR antibody. We also report morphological changes in NK cells following activation with recombinant (r)-γ IFN or r-IL 2 and the effect of TC interaction on the morphological characteristics of r-IL 2- and r-γ IFN-activated LGL. The distinctive morphological changes shown for LGL in the various experimental systems indicate that activation of NK cells and enhancement of their cytolytic ability occur

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through a series of events that include de novo formation of granules and other Golgi-related organelles, structural changes of the granule matrix, and facilitated release of the granule content.

MATERIALS AND METHODS

Cell Preparations

Human peripheral blood mononuclear cells (MNC) partially depleted of monocytes were the NK effector cell fractions in activation experiments with TC or Sepharose-bound anti-FcR antibodies (see below). MNC were isolated from heparinized peripheral blood of healthy adult volunteers by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. MNC were depleted of monocytes by density gradient centrifugation on 50.6% Percoll (Pharmacia) in RPMI 1640 with 10% fetal calf serum (RPMI-FCS) (Grand Island Biological Co, Grand Island, NY), for 15 minutes at 800 g.[27]

In other activation experiments MNC isolated as above were stimulated with r-γ IFN (Schering-Plough Corp, Bloomfield, NJ) or r-IL 2 (Biogen, Cambridge, MA) and subsequently depleted of CD3$^+$ cells using the OKT3 MoAb (Ortho Pharmaceuticals, Raritan, NJ) and nontoxic rabbit complement (Cedar Lane, Hornby, Ontario, Canada) as described.[28,29] Viable cells were recovered by Ficoll-Hypaque density gradient centrifugation.

NK Cell Activation

Lymphokines. Monocyte-depleted MNC fractions were cultured in RPMI-FCS with or without 100 IU/mL of r-IL 2 or 500 IU/mL of r-γ IFN respectively for 72 or 18 hours. Enrichment for NK cells was achieved by CD3$^+$ cell depletion following lymphokine stimulation (see above). CD3$^+$ cell-depleted preparations were further incubated overnight with RPMI-FCS containing r-IL 2 or r-γ IFN and were then processed for electron microscopy analysis.

The effect of lymphokine stimulation on NK cell-mediated cytotoxicity was tested using MNC fractions without CD3$^+$ cell depletion. The effect of r-IL 2 on the cytotoxic ability of MNC was evaluated after 6, 24, and 72 hours of culture. The effect of r-γ IFN was tested after incubation of MNC for 18 and 72 hours.

NK cell targets. To investigate the ultrastructural changes of NK cells and their programming for TC killing following interaction with K562 cells, the technique of Brahmi et al[30] was used, as detailed elsewhere.[31] Briefly, K562 cells and monocyte-depleted MNC were preselected by density gradient centrifugation on a single step gradient of 40% (by volume) 285 mOsm Percoll. K562 cells were centrifuged for five minutes at 1,200 g on the Percoll gradient, and cells at the interface were collected. MNC were centrifuged on the same gradient for three minutes at 1,200 g, and only those cells forming the pellet were collected. These preselection procedures were repeated twice to enhance the purity of the desired populations. Preselected MNC were admixed with preselected K562 cells at a 2:1 ratio, centrifuged at 200 g for seven minutes to facilitate conjugate formation, and incubated at 37°C for four hours. After incubation, all tubes received 10 mmol/L EDTA (Sigma, St Louis), were vortexed, and were vigorously pipetted to dissociate the conjugates. The dispersed MNC and TC were overlaid onto another 40% Percoll gradient and centrifuged at 1,200 g for five minutes. MNC recovered in the pellet were 99% free of K562 cells, as assessed by Wright's staining of cytocentrifuge cell preparations. These MNC cells were tested for their cytotoxic ability against K562 cells and were processed for electron microscopy analysis.

Sepharose-bound anti-FcR antibody. The AB8.28 MoAb with specificity for the FcR or for an FcR-related molecule of LGL and granulocytes was coupled with Sepharose CL-4B (Pharmacia) as described elsewhere.[19,32] For the NK cell stimulation experiments, 2×10^6 MNC were admixed with 50 μL of AB 8.28-Sepharose. This amount of antibody has been previously shown to be in excess for induction of class II antigen and IL 2-receptor expression, and for platelet-activating factor release by LGL.[19] The MNC/AB8.28-Sepharose mixture was centrifuged and incubated as a pellet for four hours at 37°C. Pellets were vigorously vortexed to dissociate conjugates, and lymphocytes were separated from Sepharose beads by centrifugation on a 40% Percoll gradient with a cell recovery of >90%. Lymphocytes in the pellet of the Percoll gradient were subsequently assayed for their cytolytic activity against K562 TC and were processed for electron microscopy analysis. Control reagents for these experiments were native Sepharose CL-4B beads and beads coated with anti-HLA-A, B, and C MoAbs. MNC incubated with these control beads were treated and assayed as cells incubated with AB8.28-Sepharose.

To determine whether NK cells treated with Sepharose-bound anti-FcR antibody retained their ability to form conjugates with K562 TC, MNC were stained with fluorescein isothiocyanate-labeled anti-Leu 11b MoAb (Becton Dickinson, Mountain View, CA) and allowed to form conjugates with TC as described elsewhere.[33] Percentages of Leu 11$^+$ (CD16$^+$) cells were also determined for these cell preparations, as well as for control cell populations.

Stimulation of lymphokine-activated NK cells with K562 TC or Sepharose-bound anti-FcR antibody. MNC cultured for 72 hours with r-IL 2 or r-γ IFN were analyzed for their cytolytic ability and for their ultrastructural features following a four-hour stimulation with K562 TC or Sepharose-bound AB8.28 antibody.

For the evaluation of cytotoxicity against K562 TC, MNC were separated from the stimulatory K562 cells using EDTA and the Percoll selection procedure described above. When Sepharose-bound AB8.28 was used as stimulator, the cytotoxicity assay was performed in the presence of the Sepharose beads. Controls for the latter experimental system were performed in the presence of similar numbers of Sepharose beads not coated with antibody. A standard ^{51}Cr-release assay was employed in these experiments (see below).

For the ultrastructural analyses, MNC were depleted of both K562 cells and Sepharose beads using EDTA and the 40% Percoll gradient described above.

Cytotoxicity Assays

Flow cytometry assay. Changes in the cytotoxic ability of NK cells after lymphokine activation or stimulation with K562 TC or Sepharose-coupled anti-FcR antibody were evaluated using a flow cytometry cytotoxicity assay described elsewhere.[34,35] Briefly, effector cells (EC) were incubated with K562 cells at EC:TC ratios ranging from 40:1 to 1.25:1 for four hours at 37°C. The EC/TC mixtures were subsequently resuspended, washed with RPMI-FCS, and stained with propidium iodide (PI, 40 μ g/mL in phosphate-buffered saline [PBS]) (Sigma Chemical Co, St Louis). In this dye-exclusion assay only nonviable cells take up PI. Cells were then analyzed by flow cytometry (FACS Analyzer, Becton Dickinson, Sunnyvale, CA) with cytometer gates set to comprise TC but to exclude EC, based on differences in cell volume and wide-angle light scatter characteristics. Two to five thousand events were collected through the appropriate cytometer gate, and the percentage of PI-stained, nonviable TC was determined. We have shown previously that this assay and the "classic" ^{51}Cr-release assay give virtually identical results.[35]

When MNC were exposed to K562 cells or Sepharose-bound anti-FcR antibody prior to the cytotoxicity assay, the percentage of recovered Leu 11$^+$ cells was determined and adjusted to that of control cell preparations. To this end MNC were stained with fluorescein isothiocyanate-labeled anti-Leu 11b MoAb and the percentage of Leu 11$^+$ cells was determined by fluorescence microscopy analysis.

^{51}Cr-release assay. MNC incubated with r-IL 2 or r-γ IFN for 72 hours and subsequently treated with K562 TC or Sepharose-bound AB8.28 MoAb (see above) were analyzed for their cytolytic ability against K562 cells using a standard ^{51}Cr-release assay.[28] MNC were admixed with ^{51}Cr-labeled target cells at ratios ranging from 40:1 to 10:1 and the % specific ^{51}Cr-release was evaluated following a four-hour incubation.

Ultrastructural Studies

Control and lymphokine-activated MNC and MNC stimulated with K562 TC or Sepharose-bound anti-FcR were fixed with 1.25% glutaraldehyde in cacodylate buffer for one hour at room temperature. Cells were rinsed in cacodylate buffer, postfixed for one hour with 1% osmium tetroxide in the same buffer, dehydrated through a graded ethanol series, and embedded in Spurr's medium.[36] Thin sections were stained with uranyl acetate and lead citrate and analyzed with a Philips 301 electron microscope.

RESULTS

Effects of r-IL 2 on LGL

Monocyte-depleted MNC cultured with or without r-IL 2 for 6, 24, and 72 hours were tested for their ability to lyse K562 TC. In accordance with that shown by others,[22] an enhancement of cell-mediated cytotoxicity was observed after a six-hour stimulation ($P < 0.5$). This enhancement was more significant ($P < 0.1$) after 24 and 72 hours of culture with r-IL 2 (Table 1). A slight progressive increase in the cytotoxic ability of control MNC was also observed, which could be due to the spontaneous release of NK cell-activating lymphokines during the culture period.

For ultrastructural analysis, MNC cultured for three days with or without r-IL 2 were depleted of CD3$^+$ cells by treatment with OKT3 MoAb and complement. The aim of this procedure was to enrich for CD3$^-$-CD16$^+$ cells and to eliminate putative cytotoxic T cells generated by r-IL 2 incubation.

Fifty percent to 70% of the CD3$^-$ cells in the control preparations displayed the morphological characteristics of resting LGL (Fig 1). These cells were round and displayed indented nuclei with clumped heterochromatin and small nucleoli. The Golgi apparatus and the centrioles were localized in the nuclear notch. Numerous electron-dense granules (five to ten per equatorial cell section) were scattered in the cytoplasm, with the majority of them containing an electron-dense matrix. Parallel tubular arrays (PTAs) and multivesicular bodies (MVB) were occasionally found. Approximately 1% of the cells displayed the features of activated LGL, as they are described below for r-IL 2-stimulated cell populations.

Following culture with r-IL 2 for three days, the majority of cells displayed changes in size, shape, and structure (Fig 2). r-IL 2-activated cells were larger than resting cells (compare magnifications in Figs 1 and 2) and assumed the shape of activated cells as shown by polar migration of the nucleus, ruffling of the cell surface, and formation of uropodia. The latter comprised most of the cytoplasmic organelles, including prominent centrioles, bundles of microtubules oriented along the longitudinal axis of the uropodium, and numerous stacks of Golgi cisternae. The cytoplasm was largely occupied by numerous smooth vesicles, some of which were seen budding from the lateral aspect of the Golgi cisternae. Granules were increased in number ($>$15 per cell section encompassing the uropodium) and size (compare Figs 1 and 2). Their matrix was highly electron-opaque and coarsely granular. MVB were found occasionally, and PTAs were virtually undetectable. LGL undergoing mitosis were also observed (Fig 2).

Table 1. IL 2-Induced Enhancement of NK Cell-Mediated Cytolytic Activity Against K562 Cells*

Hours in Culture	Control	IL 2†
6	7.1 ± 2.8‡	16.7 ± 9.1
24	9.2 ± 3.4	34.4 ± 16.3
72	18.9 ± 10.1	50.6 ± 8.3

*E:T ratio = 5:1.

†Cells were cultured with 100 IU of r-IL 2.

‡Percentages of nonviable cells as determined by flow cytometry (see Materials and Methods). Mean ± SD from six different experiments.

Effects of r-γ IFN on LGL

MNC cultured overnight with r-γ IFN displayed a significant increase in their cytolytic ability against K562 TC (Table 2).

MNC preparations stimulated with r-γ IFN were depleted of CD3$^+$ cells and further cultured overnight in the presence of r-γ IFN prior to electron microscopy analysis. Following r-γ IFN stimulation, LGL did not show those changes in size and shape observed after r-IL 2 treatment. However, the fine structure of the granules was profoundly different when compared to that of resting cells. Round cells with indented nuclei and a smooth surface were predominant, but cytoplasmic granules containing an electron-dense matrix or PTAs were virtually undetectable. These organelles were replaced by large vesicular structures containing aggregates of round vesicles or membranous myelin figures (Fig 3). A residual electron-dense matrix surrounded by small vesicles was commonly detected in multivesicular bodies. PTAs undergoing deaggregation were also observed (Fig 3). Numerous smooth and coated vesicles were free in the cytoplasmic matrix, particularly in the proximity of the Golgi apparatus which, unlike in r-IL 2-activated cells, was not expanded.

Effects of Preincubation With K562 Cells on LGL

The cytolytic ability of LGL against K562 cells was largely decreased when the effector cells were preincubated with the same TC for four hours at 37°C (Table 3). As shown in Fig 4, LGL displayed changes in shape similar to those observed for r-IL 2-stimulated cells. Uropodium formation and the rearrangement of cytoskeletal components were prominent features. Changes in the structure of the granules were also detected. Electron-dense granules and PTAs were decreased in number, and vesicles containing tubular structures, deaggregated PTAs, myelin figures, and residual

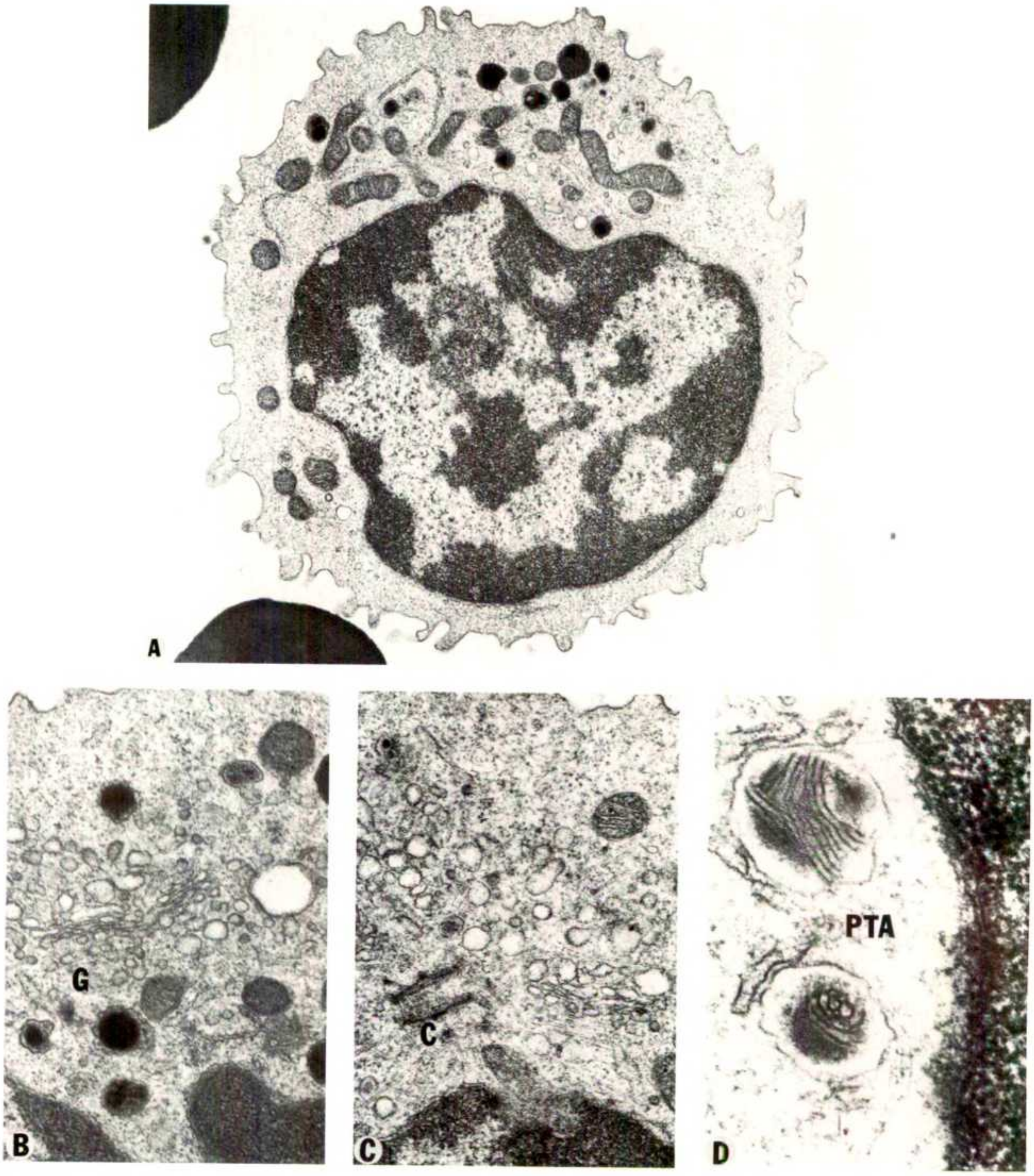

Fig 1. Ultrastructural features of resting human NK cells. (A) The majority of the electron-dense granules are localized in the nuclear notch. (B) The Golgi apparatus (G) is surrounded by granules containing an electron-denese matrix. (C) The microtubule organizing center is commonly found in resting NK cells (C, centriole). (D) Parallel tubular arrays (PTA) may be free in the cytoplasm or surrounded by unit membranes. A, original magnification ×19,500, current magnification ×12,675; B, original magnification, ×28,500, current magnification ×19,950; C, original magnification ×30,000, current magnification ×21,000; D, original magnification ×80,000, current magnification ×56,000.

matrix were commonly observed. In view of the decreased cytotoxic ability of LGL following their exposure to an NK-sensitive TC, the ultrastructural changes of the granules suggest that release of the granule content occurred under these experimental conditions.

Effects of FcR Triggering on LGL

As shown in Table 4, treatment of MNC with a Sepharose-bound anti-FcR MoAb virtually abrogated the cytotoxic ability of NK cells against K562 TC. However, NK

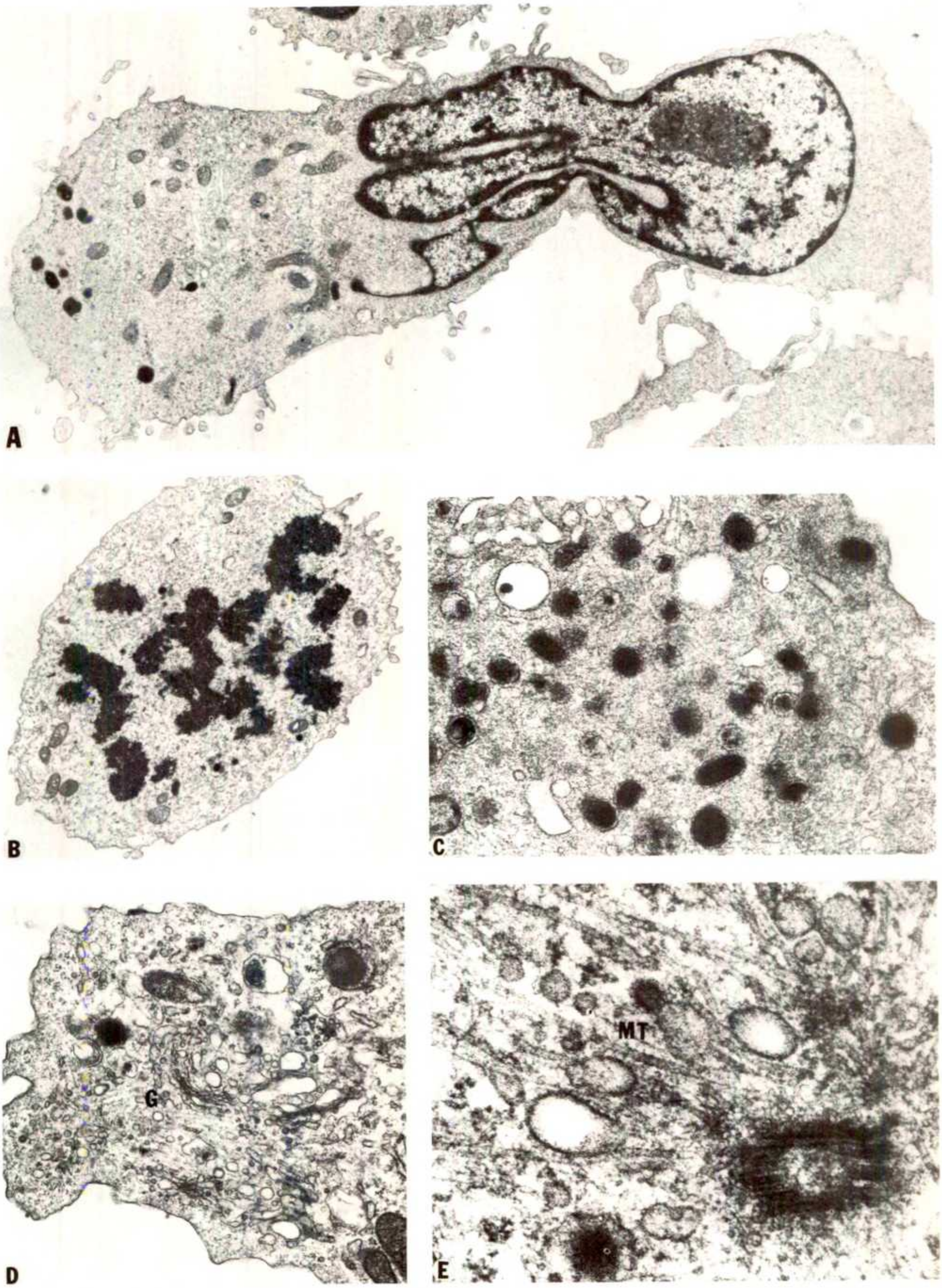

Fig 2. Ultrastructural features of r-IL 2-activated NK cells. (A) Changes in the shape of cell body and nucleus are shown. The nucleus contains a large nucleolus. Granules are confined to the uropod region. (B) A cell in mitosis still exhibits numerous electron-dense granules. (C) A cytoplasmic area displaying a high concentration of electron-dense granules. (D) The Golgi apparatus (G) is expanded, and numerous Golgi-derived vesicles are found in the uropod region. (E) Centrioles and microtubules (MT) are also found in the uropod region of r-IL 2-activated NK cells. A, original magnification, ×8,000, current magnification 5,600; B, original magnification ×10,000, current magnification ×6,200; C, original magnification ×40,500, current magnification ×18,225; D, original magnification ×35,000, current magnification ×21,700; E, original magnification ×110,000, current magnification ×49,500.

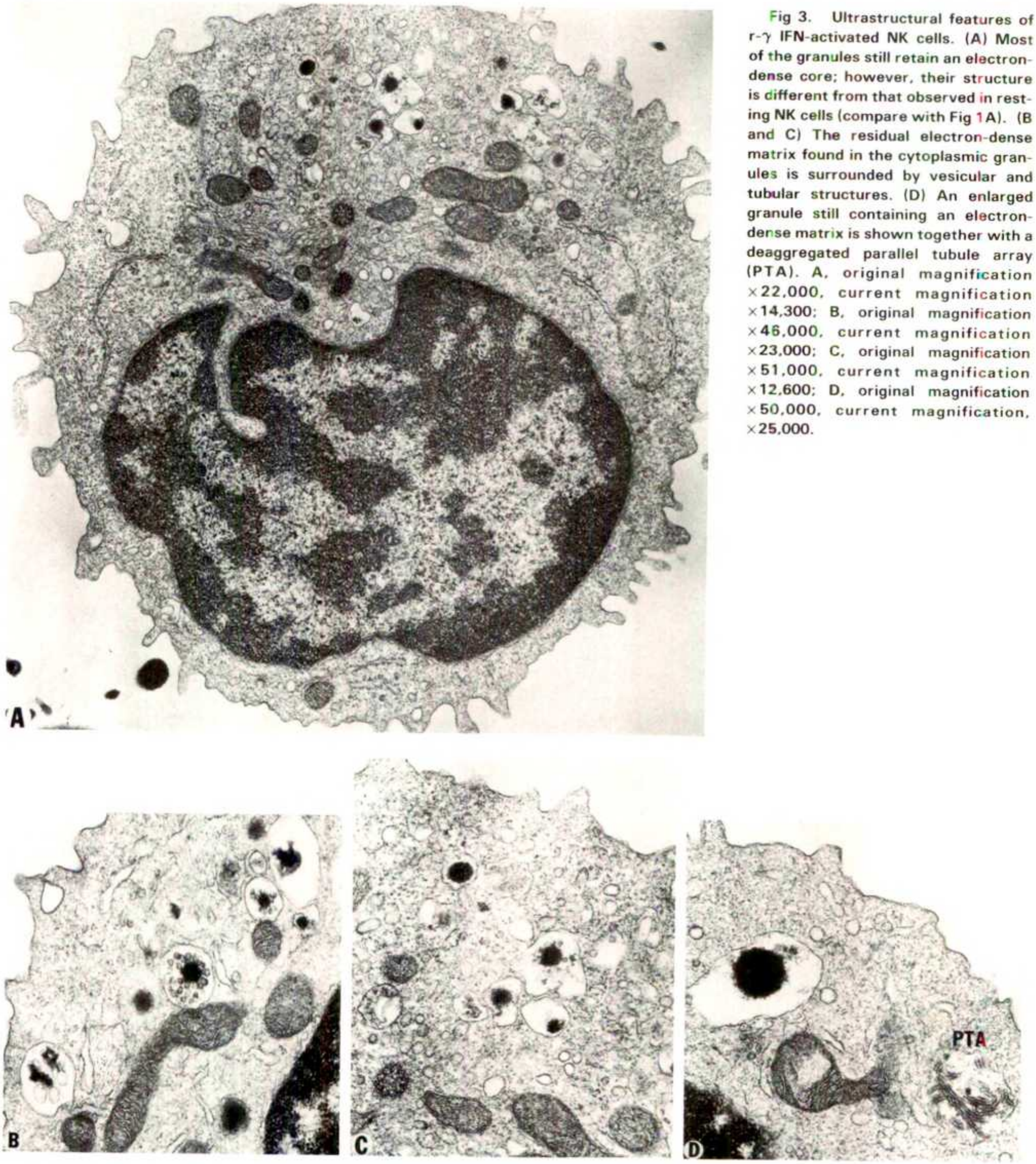

Fig 3. Ultrastructural features of r-γ IFN-activated NK cells. (A) Most of the granules still retain an electron-dense core; however, their structure is different from that observed in resting NK cells (compare with Fig 1A). (B and C) The residual electron-dense matrix found in the cytoplasmic granules is surrounded by vesicular and tubular structures. (D) An enlarged granule still containing an electron-dense matrix is shown together with a deaggregated parallel tubule array (PTA). A, original magnification ×22,000, current magnification ×14,300; B, original magnification ×46,000, current magnification ×23,000; C, original magnification ×51,000, current magnification ×12,600; D, original magnification ×50,000, current magnification, ×25,000.

Table 2. γ IFN-Induced Enhancement of NK Cell-Mediated Cytolytic Activity Against K562 Cells*

E:T Ratio	Control	γ IFN†
20:1	39.1 ± 7.1‡	52.6 ± 14.2
10:1	23.9 ± 7.1	36.0 ± 9.7
5:1	11.2 ± 2.2	17.5 ± 9.2

*Cells were incubated with r-γ IFN for 18 hours.

†500 IU of r-γ IFN/mL of culture medium.

‡% nonviable cells as determined by flow cytometry. Mean ± SD from three different experiments.

Table 3. Decrease in the Cytotoxic Ability of NK Cells by Pretreatment With NK-Sensitive Target Cells*

E:T Ratio	Untreated NK Cells	K562-treated NK Cells
40:1	51.2 ± 12.8†	25.7 ± 4.1
20:1	40.2 ± 15.3	14.5 ± 4.5
10:1	25.5 ± 12.2	8.0 ± 2.4

*See Materials and Methods section for details on the experimental procedure.

†Percentages of nonviable cells as determined by flow cytometry. Mean ± SD from four different experiments.

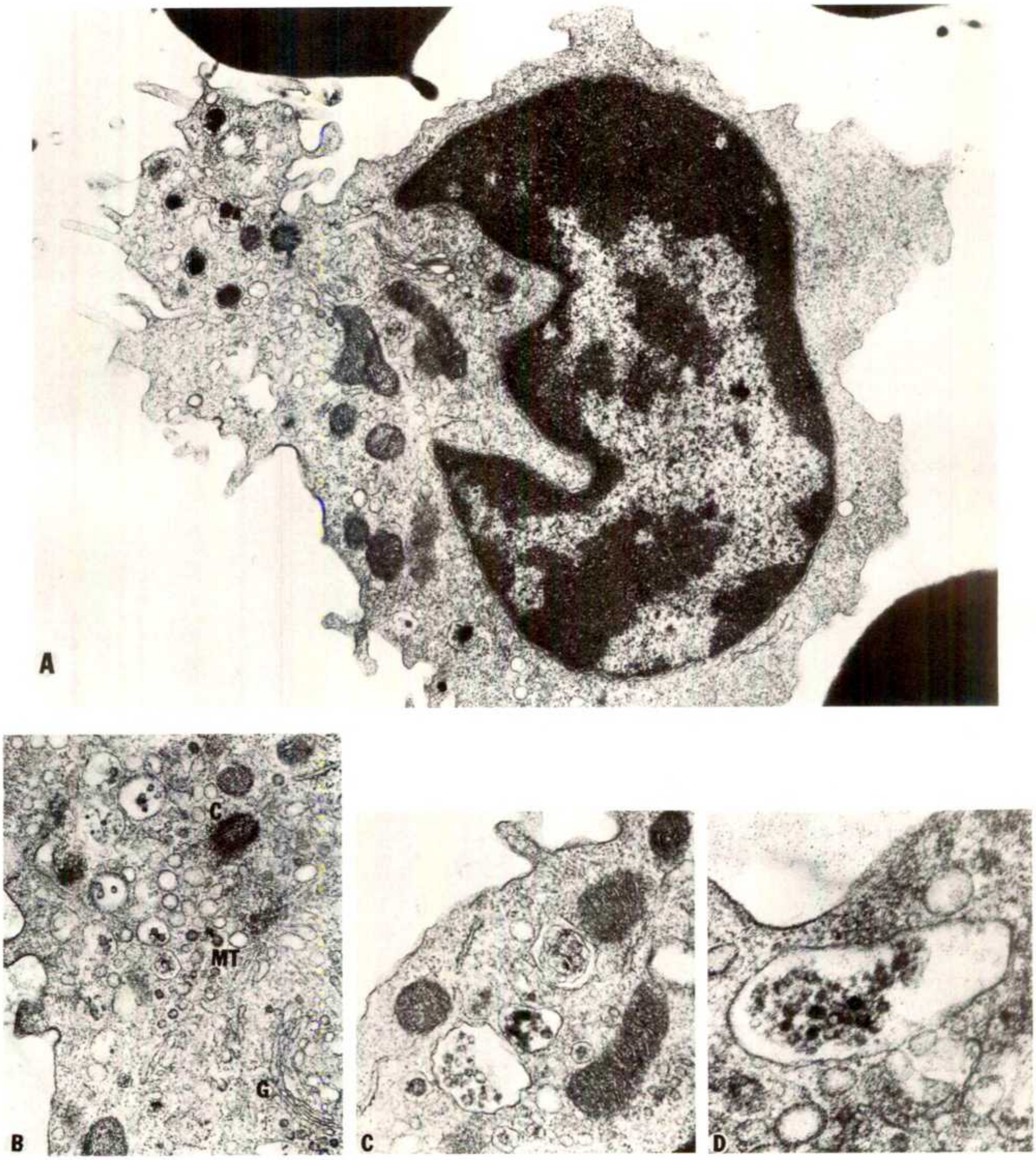

Fig 4. Ultrastructural changes of NK cells following stimulation with K562 TC. (A) Uropod formation and changes in the content of granules are shown. (B) Numerous vesicles, some of which contain tubular structures surround the microtubule organizing center (C, centriole; MT, microtubules; G, Golgi apparatus). (C and D) Changes in the content of the electron-dense granule matrix are illustrated. Electron-dense granules are replaced by vesicles containing membranous or tubular structures. A, original magnification ×22,000, current magnification ×14,300; B, original magnification ×45,000, current magnification ×24,750; C, original magnification ×51,000, current magnification ×28,050; D, original magnification ×110,000, current magnification ×60,500.

Table 4. Decrease in the Cytotoxic Ability of NK Cells by Pretreatment With Sepharose-Bound Anti-FcR Antibodies*

E:T Ratio	Untreated Cells†	AB8.28-Sepharose-Treated Cells†
40:1	25.3 ± 11.4‡	3.6 ± 1.5
20:1	12.6 ± 5.0	2.3 ± 1.1
10:1	9.0 ± 4.7	0.8 ± 0.2

*See Materials and Methods section for details on the experimental procedures.

†Percentages of Leu 11⁺ cells were 11.5 ± 2.3 and 12.4 ± 4.2 for untreated and for AB8.28-Sepharose-treated cell preparations, respectively.

‡Percentages of nonviable cells as determined by flow cytometry. Mean ± SD from three different experiments.

cells did not lose the ability to form conjugates with TC because the absolute percentage of Leu 11^+ cells (Table 4) and the percentage of Leu 11^+ cells bound to K562 TC (81% and 90% respectively) were similar for AB8.28-Sepharose-treated MNC as for control MNC. In contrast, Sepharose-bound anti-HLA-A, B, C antibodies and uncoated Sepharose beads did not produce any significant change in the cytolytic ability of MNC or in their ultrastructural features (not shown).

When examined by electron microscopy, the majority of anti-FcR-stimulated MNC displayed features of small or medium lymphocytes. Granules, vesicular structures, and PTAs were no longer detectable in >99% of the cells. However, persistence of other typical morphological features such as nuclear indentation, a well developed Golgi apparatus and centrioles, or residual granule-related structures allowed identification of "agranular" LGL (Fig 5). Less than 1% of the cells demonstrated uropod formation and

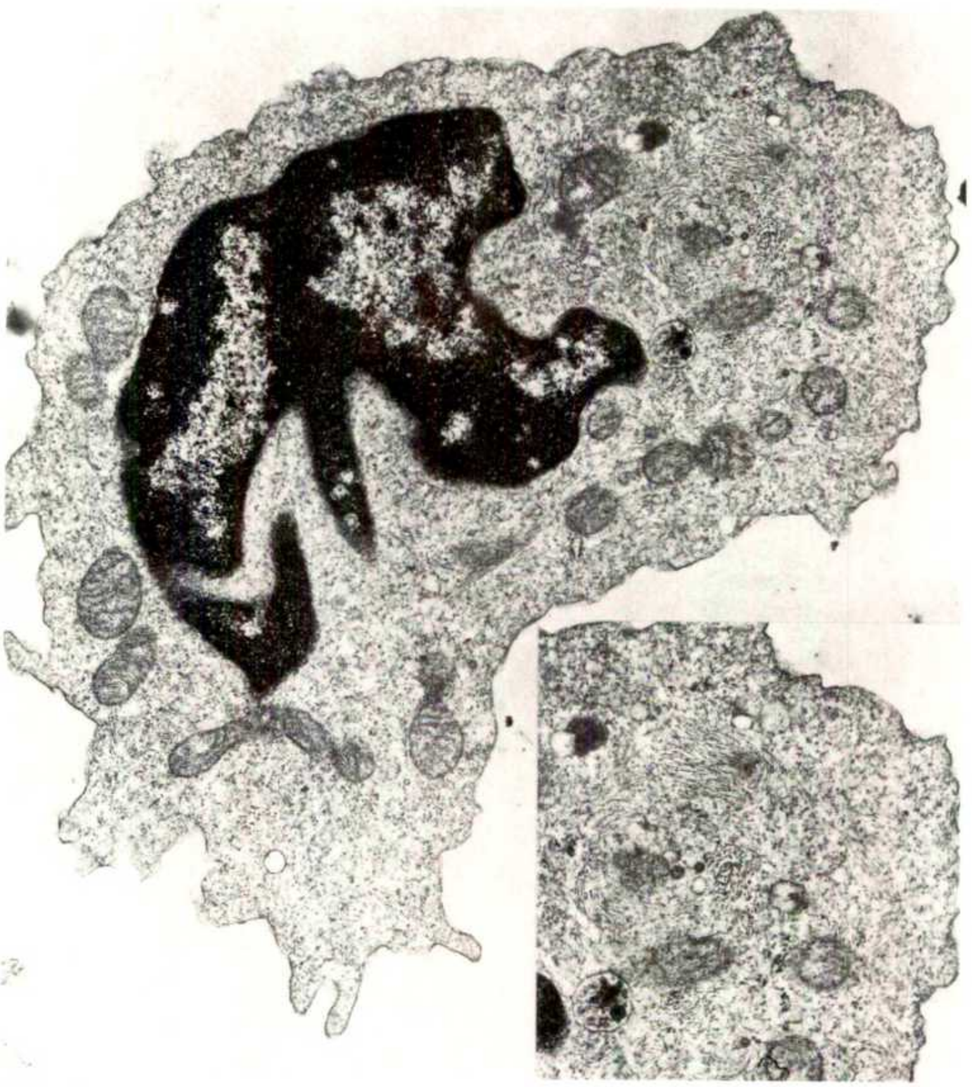

Fig 5. Ultrastructural changes of NK cells following stimulation with Sepharose-bound anti-FcR antibody. The cell is largely depleted of electron-dense granules. Residual multivesicular bodies and prominent microfilament bundles are shown in the inset. Original magnification ×25,000, current magnification ×18,750. Inset, original magnification ×30,000, current magnification ×22,500.

Table 5. Decrease in the Cytotoxic Ability of r-IL 2- and/or r-γ IFN-activated Cells by Stimulation With Sepharose-Bound Anti-FcR Antibody (AB8.28) or With K562 Cells

		Effector to Target Ratios*		
Lymphokine	Stimulator	40:1	20:1	10:1
None	None	45.0 ± 7.7†	40.1 ± 12.7	29.1 ± 12.0
r-IL 2	None	68.3 ± 3.7	68.0 ± 1.9	68.3 ± 2.0
r-IL 2	AB8.28	35.4 ± 25.0(48)‡	31.7 ± 24.0(53)	22.7 ± 19.7(67)
r-IL 2	K562	44.3 ± 17.8(35)	35.0 ± 18.5(49)	23.6 ± 13.8(65)
r-γIFN	None	62.5 ± 5.2	57.2 ± 11.6	48.7 ± 18.2
r-γIFN	AB8.28	7.7 ± 6.0(88)	3.6 ± 2.5(94)	1.4 ± 1.1(97)
r-γIFN	K562	36.0 ± 20.5(42)	23.0 ± 14.2(60)	12.7 ± 8.0(74)

*Using a ^{51}Cr-release assay with K562 target cells.

†Means ± SD of % specific ^{51}Cr-release from three different experiments.

‡Data in parentheses are the average percentual decrease of specific ^{51}Cr-release in comparison with lymphokine-activated, unstimulated cells.

changes in the structure of the granules similar to those induced by r-γ IFN or TC treatment. These ultrastructural observations indicate that LGL lose their complement of cytoplasmic granules following exposure to Sepharose-bound anti-FcR antibodies and provide an explanation for the complete abrogation of NK cell-mediated cytotoxicity observed in these experimental conditions.

Effects of Stimulation by K562 TC or Sepharose-Bound Anti-FcR Antibody on Lymphokine-Activated LGL

The ultrastructural changes described above for r-IL 2- or r-γ IFN-activated LGL have been related to their enhanced cytolytic ability. However, it is possible that increased granulogenesis and changes in the structure of the granule matrix are associated with other LGL functions, such as release of γ IFN or other secretory products. To reinforce the relationship between the structure of lymphokine-activated LGL and their increased killing efficiency, MNC were cultured for 72 hours with r-IL 2 or r-γ IFN and subsequently stimulated with K562 TC or Sepharose-bound AB8.28 antibody, two types of stimuli that are effective in reducing or abrogating the cytolytic ability of resting NK cells (see above).

The cytotoxic ability of LGL activated by both lymphokines was largely reduced following stimulation with K562 TC or Sepharose-bound anti-FcR antibody (Table 5). At the ultrastructural level both types of stimuli produced a complete degranulation of lymphokine-activated cells. A notable finding was that r-IL 2-activated cells stimulated by K562 TC or Sepharose-bound AB8.28 antibody displayed intracytoplasmic fusion of granules followed by a massive discharge of the content of multiple granules (Fig 6). These morphological patterns of granule discharge resemble those observed in basophils triggered to degranulate via cross-linking of membrane ligand molecules or other types of stimuli.[37,38] A complete degranulation was also observed in r-γ IFN-activated LGL (not shown).

DISCUSSION

It has been repeatedly shown that IL 2 or γ IFN activate natural cytotoxic cells in vitro and in vivo,[20-26,39,40] but mechanisms underlying this functional enhancement are not yet eludicated.

This study provides a morphological basis for the mechanisms of the enhanced NK cell-mediated cytotoxicity following stimulation of peripheral blood MNC with r-IL 2 or r-γ IFN. It also describes ultrastructural changes occurring in resting or lymphokine-activated LGL after their interaction with NK-sensitive TC or after triggering of surface FcR.

CD3$^-$ LGL displayed distinctive structural changes following three-day culture with r-IL 2. Morphological features indicated increased cellular motility, de novo granule formation, and cell proliferation. The Golgi apparatus was increased in size and appeared to release vesicles that were particularly numerous in the uropodium region. Thus the r-IL 2-mediated increase of NK cytotoxicity could be explained by the availability of a higher complement of cytoplasmic granules and vesicles and by an increased rate of cytotoxic factor release due to enhanced activity of cytoskeletal structures associated with export of granule and vesicle contents. The association of these ultrastructural changes with augmentation of NK function was reinforced by observation of a massive degranulation of r-IL 2-activated LGL upon contact with K562 TC.

MNC preparations cultured with r-γ IFN and subsequently enriched for CD3$^-$ cells displayed ultrastructural features different from those of r-IL 2-activated cells. No changes in cell size, shape, or number of granules were observed, but a dramatic change in the structure of the granules was noted. The electron-dense granules and PTAs commonly found in resting LGL were replaced by larger membrane-bound organelles containing vesicles, deaggregated PTAs, and a residual electron-dense matrix. Several studies have suggested that vesicular or tubular structures found in the cytoplasm of activated cytotoxic cells represent the morphological equivalent of cytolytic factors.[10,11,38,41-43] Such structures are generated following interaction of the effector cells with the appropriate target cells and are detected within membrane-bound vesicles that are apparently derived from electron-dense granules.[41-43] Our observations demonstrate the appearance of similar structures following stimulation of CD3$^-$ LGL with r-γ IFN and suggest that the enhanced cytolytic ability induced by this lymphokine may reflect a preprogramming of the LGL via changes in their granule content. These changes may lead to the ready availability of active cytotoxic factors.

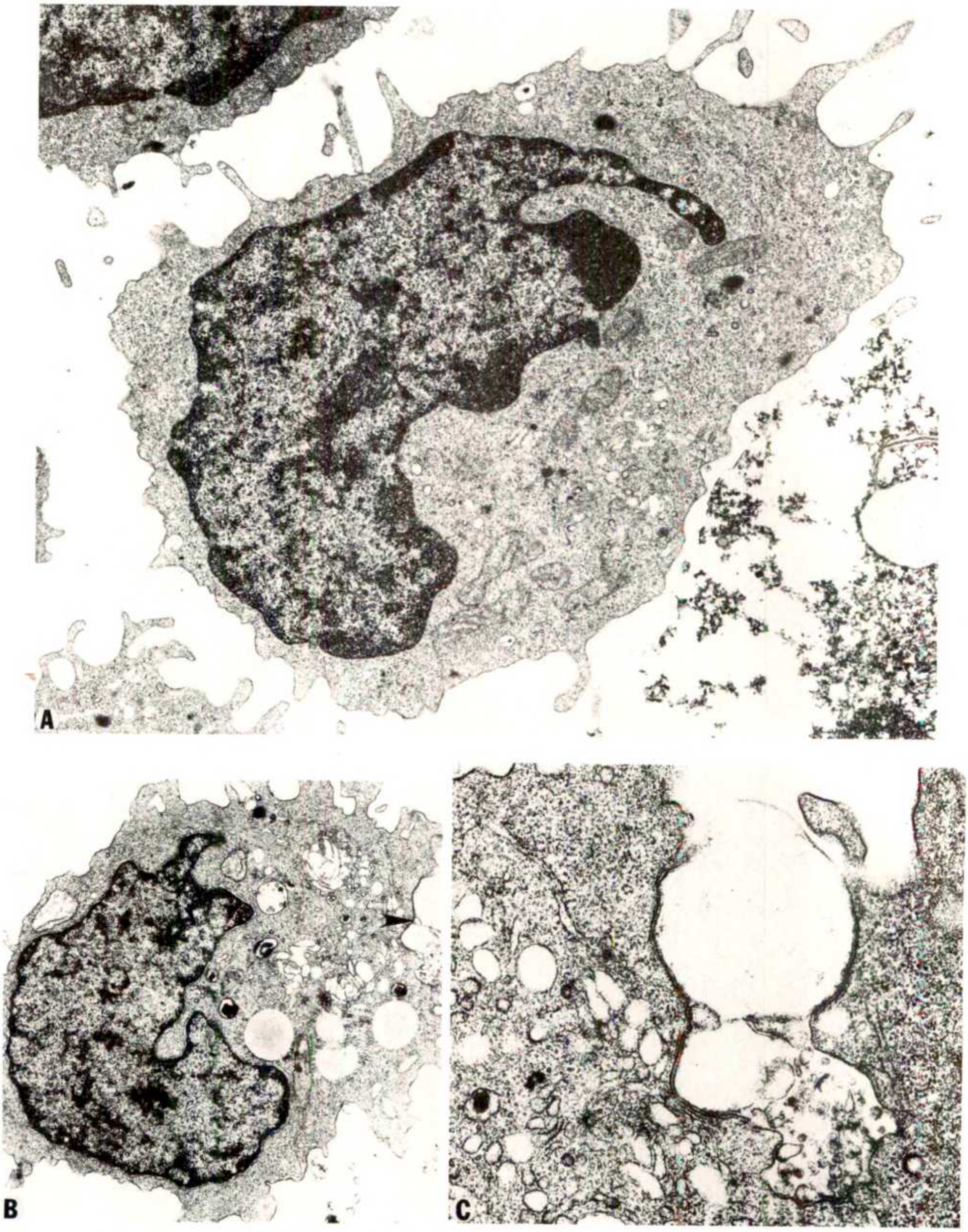

Fig. 6. Ultrastructural features of r-IL 2-activated NK cells following stimulation with K562 TC. (A) A large, activated cell is virtually devoid of electron-dense granules. (B) Another cell in the same experimental system exhibits intracytoplasmic fusion of granules. Arrowhead indicates the structure illustrated in C. (C) Ultrastructure of channels formed by fusion granules that release their content extracellularly. Residual tubular structures are found within the channel formed by granule fusion. A, original magnification ×16,300, current magnification ×10,595; B, original magnification ×9,500, current magnification ×6,650; C, original magnification ×57,000, current magnification ×39,900.

Ultrastructural studies and the immunofluorescence localization of cytoskeletal components (eg, actin, tubulin, and vinculin) have shown that following interaction with the appropriate TC, cytotoxic cells acquire the shape of activated cells, redistribute their cytoskeletal structures, and display marked changes in their granule content.[10,13-17] Our observations confirm these findings by showing that following conjugate formation with K562 cells, LGL form uropodia and exhibit prominent microtubules and marked changes in the content of their granules. Changes in shape and cytoskeletal reorganization are similar to those documented for r-IL 2-stimulated cells, whereas changes in the granule content are identical to those shown for r-γ IFN-stimulated cell and also described by others for both NK cells and cytotoxic T cells.[41-43] Therefore the synergistic effect on NK cell-mediated cytotoxicity noted with the combined use of r-IL 2 and r-γ IFN[44-46] can be explained by the action of the two lymphokines on two distinct cellular machineries. In addition, our functional studies provide evidence that, following interaction with sensitive TC, LGL release cytolytic factors, resulting in their impaired ability to kill K562 cells upon successive exposure to TC.[47] All of the above ultrastructural observations demonstrate that NK cells respond to a variety of stimuli (lymphokines, triggering of surface membrane ligands, conjugate formation with tumor cells) by showing distinctive structural changes. The resulting increased cytolytic ability and the initiation of the cytolytic program are the result of increased granule formation, changes in the granule structures, and reorganization of cytoskeletal structures that may facilitate the release of the granule content.

The AB8.28 MoAb is able to block FcR-mediated functions of NK cells and granulocytes, such as erythrocyte-antibody (EA) rosette formation, IgG-mediated phagocytosis, and ADCC.[32] However, the antigen recognized by AB8.28 does not comodulate with the FcR (CD16) identified by other MoAbs (eg, Leu 11, VEP 13, B73.1),[48] and it could be an FcR-associated structure or a distinct type of FcR. Sepharose-bound AB8.28 activates LGL as indicated by expression of class II antigens and IL 2 receptors and release of platelet-activating factor by LGL treated with this reagent.[19] Here we have shown that the cytolytic ability of both resting and lymphokine-activated LGL is virtually abrogated after their exposure to Sepharose-bound AB8.28. This is not due to the lack of conjugate formation but to complete discharge of granules from AB8.28-treated LGL. Similar results have been obtained using Sepharose-bound anti-CD16 MoAbs, such as B73.1 (Leu 11c) (B. Perussia and C.E. Grossi, manuscript in preparation). Thus it appears that triggering of FcR or of an FcR-related structure may deliver an activation signal to LGL mimicking that provided by the TC.

In conclusion, this study has shown that r-IL 2 and r-γ IFN enhance NK cell-mediated cytolysis by mechanisms that result in different ultrastructural changes of LGL. Furthermore, activation of NK cells and release of cytotoxic factors shown after TC or FcR stimulation suggest that different surface membrane molecules may trigger the activation pathway of LGL. The same signals appear to be effective in the triggering of both resting and lymphokine-activated LGL.

ACKNOWLEDGMENT

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Type β Transforming Growth Factor Is a Potent Inhibitor of Murine Megakaryocytopoiesis In Vitro

By Toshiyuki Ishibashi, Susan L. Miller, and Samuel A. Burstein

To investigate the potential role of platelets in the inhibition of megakaryocytopoiesis, freeze-thawed extracts of human platelets were added to serumless liquid cultures of murine marrow. When acetylcholinesterase (AchE), a marker of megakaryocytic differentiation in mice, was assayed, a significant inhibition of enzymatic activity was noted in cultures containing the equivalent of greater than 5 × 10^6 solubilized platelets per milliliter. Freeze-thawed extracts of granulocytes had significantly less inhibitory effect than did platelets. Transforming growth factor β (TGF-β), a growth factor known to be inhibitory to some cell lineages and to be found at relatively high concentrations in platelets, was then added to liquid marrow cultures. A similar inhibition of AchE activity was detected when cultures were stimulated with mitogen-stimulated conditioned medium. The effect was potent with 50% inhibition of AchE activity observed at 4 pmol TGF-β/L. To determine if TGF-β inhibited specifically one aspect of megakaryocytic differentiation, the factor was added to isolated single megakaryocytes in serumless culture induced by interleukin 3 (IL3) to increase in size. The number of megakaryocytes increasing in size in response to IL 3 exposure was reduced from 68% to 20% when both factors were simultaneously added to cultures. Colony assays showed that megakaryocytic and granulocyte-macrophage colony detection was inhibited at picomolar concentrations of the factor. These data suggest that TGF-β is a potent in vitro inhibitor of the murine megakaryocytic lineage, although its effects are not limited to this lineage.

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PROLIFERATION of the human megakaryocytic colony-forming cell (CFU-MK) has been shown by some investigators to be enhanced by the use of plasma rather than serum as a culture constituent.[1-4] The reasons for this occurrence may be in part related to inhibitory factors derived from platelets during the preparation of serum. Although serum rather than plasma has been used routinely in the culture of murine megakaryocytic colonies with no obvious adverse influence, this area has not been carefully studied. We recently observed that extracts of human platelets inhibited megakaryocytopoiesis in liquid cultures of murine marrow. Since platelet extracts have been shown to be a source of transforming growth factor β (TGF-β),[5] we hypothesized that this polypeptide might be the inhibitor or one of several inhibitors of megakaryocytopoiesis derived from platelets. TGF-β is a 25,000 mol wt homodimer found in numerous tissues; however, platelets appear to be the richest known source of this material.[5-7] TGF-β is a unique polypeptide growth factor, promoting growth of some cell lines while inhibiting others.[8-12] In this report, we show that TGF-β inhibits in vitro murine megakaryocytopoiesis, although its effects are not limited to this lineage.

MATERIALS AND METHODS

Preparation of platelet and granulocyte extracts. Blood was obtained from normal human volunteers and collected into 0.2 volumes of ACD. Platelet-rich plasma was prepared by two centrifugations at 200 g for ten minutes at room temperature. Following adjustment of the pH to 6.5 with 0.15 mol/L citric acid, the platelets were then pelleted by centrifugation at 1,200 g for 15 minutes. The pellets were resuspended into 1 mL of a buffer containing 0.129 mol/L NaCl, 8.6 mmol/L Na_2HPO_4, 1.6 mmol/L KH_2PO_4, 13.6 mmol/L Na citrate, 11.1 mmol/L glucose, pH 6.5, and gel-filtered through Bio-Gel A-50m (Bio-Rad Laboratories, Richmond, CA) to deplete contaminating plasma proteins. Seventy percent to 80% of the applied platelets were recovered following gel filtration. Platelets were adjusted to 10 × 10^8/mL with phosphate-buffered saline (PBS) and freeze-thawed four times. Granulocytes were prepared by dextran sedimentation followed by centrifugation through Ficoll-Paque (Pharmacia, Piscataway, NJ) as follows[13]: 30 mL of human blood anticoagulated with 17% volume ACD was mixed with 20 mL of Macrodex (6% dextran 70 in normal saline; Pharmacia) in a 60-mL syringe. The syringe was maintained upright for one hour at room temperature. Following sedimentation the straw-colored upper layer was removed carefully and centrifuged at 150 g for ten minutes at 4°C. The pellet was treated with ice-cold H_2O to lyse red cells followed by the addition of 0.6 mol/L NaCl. After centrifugation at 150 g for ten minutes, the cells were overlaid on Ficoll-Paque and centrifuged at 300 g for 20 minutes at 4°C. The pellet was washed with PBS, adjusted to 2 × 10^7 cells/mL, and finally freeze-thawed four times. This preparation contained >99% granulocytes. After centrifugation at 15,000 g for five minutes, the supernatants were removed and the protein concentration was measured, followed by dilution to the desired concentrations with PBS. The protein concentrations of the extracts were measured by the method of Bradford.[14]

Mice. Six- to eight-week-old specific pathogen-free $C_{57}Bl/6$ male mice obtained from Jackson Laboratories, Bar Harbor, ME, were used for all experiments.

Marrow cell preparation. Mice were killed by cervical dislocation. The femurs were removed and the marrow expelled with 4 mL of Iscove's modification of Dulbecco's medium (IMDM; Irvine Scientific, Irvine, CA). A monocellular suspension was made by repetitive expulsion through progressively smaller needles. Cells were washed by centrifugation in IMDM at 250 g for ten minutes at 4°C. Viable cell counts were performed by trypan blue exclusion.

Liquid marrow cultures. Marrow cells were treated with 0.5 mmol/L diisopropylfluorophosphate in 100% ethanol for 20 minutes to inactivate endogenous cholinesterase.[15] Cells (1 × 10^5 per well) were cultured in microwell plates in a final volume of 0.2 mL IMDM

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supplemented with 1% Nutricyte (J. Brooks Laboratories, San Diego), a serum-free medium supplement containing 2% pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) produced under serum-free conditions and various concentrations of platelet extract or transforming growth factor-beta (TGF-β; 96% pure; R & D Systems Inc, Minneapolis). One microgram of TGF-β was reconstituted in 1 mL of 4 mmol/L HCl and diluted to the desired concentrations with 4 mmol/L HCl containing 50 μg/mL of crystalline bovine serum albumin (BSA; Sigma Chemical Co, St. Louis). Control wells contained either the cellular extract diluent (PBS) or TGF-β diluent (4 mmol/L HCl + 50 μg/mL BSA).

Acetylcholinesterase (AchE) assay. AchE activity of liquid marrow cultures was measured fluorometrically to quantitate megakaryocytopoiesis.[15,16] After four days of culture, the cells were centrifuged at 700 g for ten minutes, and the supernatant was discarded. Two-tenths milliliter of a solution of 0.2% Triton X-100 in 1 mmol/L EDTA, 0.12 mol/L NaCl, and 50 mmol/L HEPES pH 7.5 was added to each well followed by the addition of 20 μL of 5.9 mmol/L acetylthiocholine iodide. After four hours of incubation, 10 μL of 0.4 mmol/L coumarinphenylmaleimide (Molecular Probes Inc, Junction City, OR) was added to 10 μL of the reaction mixture in a borosilicate glass tube followed by 2 mL of 5 mmol/L sodium acetate pH 5.0 containing 1 mmol/L EDTA and 0.2% Triton X-100. The fluorescence emission was measured with a filter fluorometer with an excitation filter of 390 nm and an emission filter of 450 nm.

Single cell culture of CFU-MK-derived megakaryocytes. Marrow cells were first separated on a 1.070/1.077 g/cm^3 discontinuous Percoll gradient to concentrate CFU-MK. One to 2 × 10^4 cells were cultured in 1% methylcellulose containing 15% platelet-poor plasma (PPP)-derived horse serum (Hyclone Laboratories, Logan, UT) and 5% PWM-SCM. After five days, megakaryocytic colonies were identified in situ and were selectively removed with a micropipette in 2 μL volumes and dispersed into 2 mL of IMDM containing 50 μmol/L β-mercaptoethanol (β-ME). Fewer than five colonies were removed at a time to maximize the dilution of the initial culture constituents. Individual cells were removed in 1-μL volumes with a 20-μL adjustable automatic pipette (Gilson Pipetman; Rainin Instrument Co, Emeryville, CA) and cultured with IL 3 (5 U/mL; provided by Dr James Ihle), TGF-β (20 pmol/L), or both in 35 μL final volume containing 3% BSA, 1% Nutricyte, and 50 μmol/L β-ME for 24 hours as previously described.[17] Cell diameter was measured by determining the geometric means of two perpendicular diameters using an ocular sizing grid calibrated with a stage micrometer under an inverted microscope.[18] Cells of initial diameter of 12 to 25 μm were chosen for this study.

Colony assays. To assess the influence of TGF-β on the detection of megakaryocytic and granulocyte-macrophage (CFU-GM) colony-forming cells, marrow cells were cultured at 1 × 10^5 cells/mL in agar in 1-mL final volume in 35 mm Lux tissue culture dishes as previously described, with the exception that horse serum was replaced with horse plasma-derived serum to presumably minimize TGF-β contamination.[19] PWM-SCM was used as a source of colony-stimulating activity. CFU-MK and CFU-GM-derived colonies were enumerated in situ.[19] Three to five replicate plates were counted for each experiment. TGF-β was added to each well at concentrations ranging from 0.4 to 800 pmol/L.

Statistical analysis. Statistical analyses were performed by Student's *t*-test and chi-square analysis. Comparison between platelet and granulocyte extracts was performed by log-logit transformation of the raw data to linear plots and subsequent linear regression analysis of the differences between slopes.

RESULTS

Effect of cellular extracts on AchE activity in liquid culture. AchE activity was assayed fluorometrically on whole marrow cells treated with PWM-SCM and various concentrations of platelet extracts. As shown in Fig 1, platelet extracts inhibit AchE activity in murine liquid marrow cultures. A significant reduction was observed employing ≥5 × 10^6/mL of solubilized platelets ($P < 0.05$). This decrease in AchE activity was accompanied by a decrease in the size and apparent number of megakaryocytes when observed microscopically. Figure 2 shows the inhibition of AchE production of platelet *v* granulocyte extracts. A log-logit transformation was performed to linearize the data, as other means of representing the data yielded curvilinear plots. Platelet extract induced a significantly greater degree

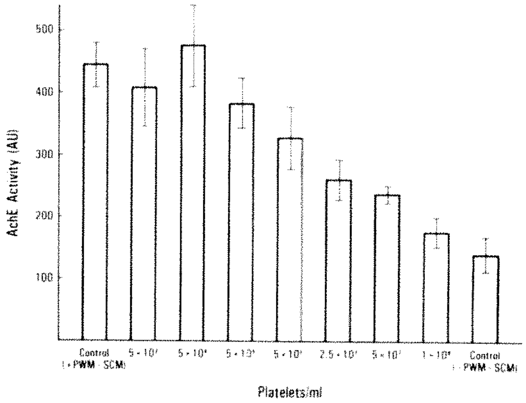

Fig 1. The effect of freeze-thawed platelet extracts on AchE activity in liquid marrow cultures stimulated with PWM-SCM. A significant inhibition of AchE activity is observed at a concentration ≥5 × 10^6 platelets/mL ($P < 0.05$; Student's *t* test). One of six similar experiments. The error bars represent ± 1 SD of five replicate wells. AU, arbitrary fluorescence units.

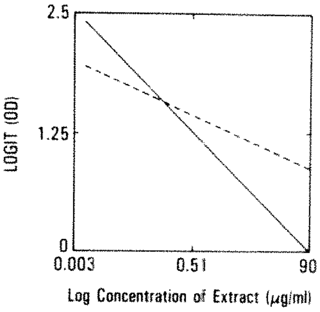

Fig 2. Comparison of inhibitory activity of freeze-thawed platelet *v* granulocyte extracts. Extracts prepared as described in the Materials and Methods section. The abscissa represents the log of the protein concentration, while the ordinate shows the logit (log{[% of control OD]/100 − % of control OD]}), representing the AchE activity. A logit of zero thus indicates 50% inhibition. The slopes of these computer-generated lines are significantly different ($P < 0.001$; linear regression analysis). The solid line (platelet extract) is a composite of six experiments (each experiment was done with five different protein concentrations; at each concentration five replicate wells were assayed), while the dashed line (granulocyte extract) is a composite of four experiments done identically. Fifty percent inhibition of AchE activity is observed with 90 μg/mL of platelet extract. None of the tested concentrations of granulocyte extract induced 50% inhibition. Twenty-eight percent inhibition was observed at 90 μg/mL of granulocyte extract.

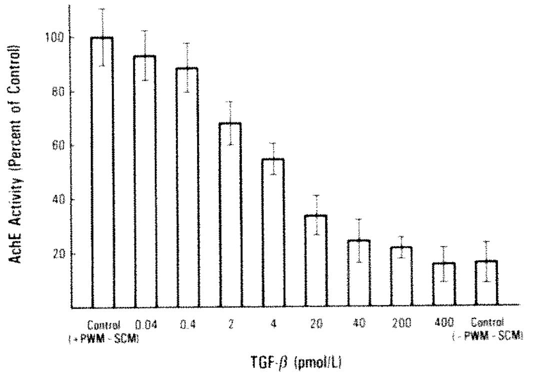

Fig 3. The effect of TGF-β on AchE activity in liquid marrow cultures stimulated with PWM-SCM. A significant inhibition of AchE activity is observed at 2 pmol/L TGF-β ($P < 0.05$; Student's *t* test). The data represent the mean ±1 SD of six experiments. Five replicate wells were assayed for each experiment.

of inhibition than an equivalent amount of granulocyte extract ($P < 0.001$).

Influence of purified TGF-β on AchE activity in liquid culture. Figure 3 shows a significant decrease in AchE activity at a concentration of 2 pmol/L TGF-β, with further reductions at higher concentrations ($P < 0.05$). Four pmol/L TGF-β was required for 50% inhibition of AchE activity. Visual inspection of these cultures showed a decrease both in the size and apparent number of megakaryocytes when compared to control cultures. When IL 3 was substituted for PWM-SCM, the identical inhibitory effects of TGF-β were observed (data not shown). To determine whether the inhibition of AchE activity was due to cytotoxicity of TGF-β, cell viability after culture was measured by trypan blue exclusion. No significant difference was observed between cells cultured with or without 400 pmol/L TGF-β ($P < 0.01$).

Cell diameter of single megakaryocytes isolated from CFU-MK exposed to TGF-β. Single megakaryocytes were cultured with 5 U/mL IL 3, 20 pmol/L TGF-β, or both factors at the indicated concentrations. Cell diameter was measured on 123 megakaryocytes before and after 24 hours of culture. As shown in Table 1, 68% of the cells increased in diameter in response to IL 3, whereas only 20% of the cells increased in response to the combination of IL 3 and TGF-β. The response to IL 3 was significantly inhibited by TGF-β ($X^2 = 14.6$, $P < 0.0001$).

Colony assays. CFU-MK and CFU-GM were enumerated in situ after six days of culture. The effect of TGF-β on progenitor cells is shown in Table 2. No change in the numbers of CFU-MK- and CFU-GM-derived colonies were detected at concentrations ≤4 pmol/L. Inhibition of colony formation by both progenitor cell types was observed at concentrations ≥20 pmol/L ($P < 0.01$). However, at maximum inhibitory concentrations, CFU-MK numbers were decreased by 77%, whereas CFU-GM were decreased by 50%. Moreover, the numbers of cells per megakaryocyte colony and the size of the individual megakaryocytes within colonies were decreased in cultures containing TGF-β (data not shown).

Table 1. The Effect of TGF-β on Isolated Single Megakaryocytes

	Number of Cells Increasing in Diameter (%)
Control	5/34 (15)
IL 3 (5 U/mL)	15/22 (68)
TGF-β (20 pmol/L)	3/26 (12)
IL 3 (5 U/mL) + TGF-β (20 pmol/L)	8/41 (20)

An increase in diameter was defined as an increment of greater than 0.5 μm. The range of increments was 0.5 to 7.2 μm. The data represent the summation of all cells analyzed in five experiments. The difference between cells treated with IL 3 + TGF-β and IL 3 alone is highly significant ($P < 0.0001$; chi-square analysis).

DISCUSSION

Although inhibitors of hematopoiesis have been postulated for many years, it has only been recently demonstrated that specific purified factors such as lactoferrin and acid isoferritins inhibit growth factor-driven hematopoietic cellular proliferation.[20-23] Suggestions that megakaryocytic proliferation might be subject to a significant inhibitory mechanism were provided by the studies of Messner et al,[1] Vainchencker et al,[2] Kimura et al,[3] and Solberg et al.[4] These investigators observed that megakaryocytic colony growth was markedly improved when the culture medium was supplemented with human plasma rather than serum and that the inhibitory activity appeared to be derived from platelets.

To determine if a platelet-derived factor might inhibit murine megakaryocytopoiesis, we added freeze-thawed extracts of human platelets to serumless liquid cultures of murine marrow. We measured AchE, a relatively specific marker of the murine megakaryocytic lineage by a fluorometric method that is sensitive to low concentrations of the enzyme.[17,24,25] Measurement of enzyme activity presumably reflects both the number and the state of differentiation of megakaryocytes.[15,16] At concentrations of extract equivalent to 5 × 10^6 platelets per milliliter, a significant inhibition of AchE activity was noted. These data together with the human colony data are somewhat at odds with the findings of Williams et al.[26] These workers could detect no effect of the addition of freeze-thawed platelet extracts on the numbers of murine megakaryocytic colonies detectable in agar cultures. It is possible that the use of fetal calf serum or the conditioned medium used by Williams et al might account for the differences observed.

Table 2. Effect of TGF-β on Colony Growth

TGF-β (pmol/L)	CFU-MK/10^5 Cells	CFU-GM/10^5 Cells
0	22 ± 2	123 ± 13
0.4	19 ± 2	107 ± 9
4	19 ± 2	101 ± 16
20	15 ± 3	95 ± 8
40	12 ± 3	76 ± 7
100	6 ± 1	73 ± 11
200	5 ± 2	70 ± 13
400	6 ± 2	61 ± 10
800	(7)	(76)

The data represent the mean ± 1 SD of six experiments except for the numbers in parentheses (one experiment). Three to five replicate plates were enumerated for each experiment. A significant difference was observed at 20 pM TGF-β for both CFU-MK and CFU-GM ($P < 0.01$; Student's *t* test).

However, studies of inhibition employing whole cell extracts are complex. Inhibition may be specific and have significance with respect to regulation. Conversely, inhibition may be nonspecific or trivial and related to proteolysis, pH changes, or other potential effects of whole cell extracts. Since extracts of cells other than platelets probably contain numerous constituents not present in platelet extracts, appropriate controls for this type of experiment are difficult to devise. On a per weight basis, however, platelet extracts contain more inhibitory activity than granulocyte extracts. Nevertheless, a more convincing experiment might be the documentation of inhibition by a purified factor at concentrations physiologically achievable.

Recently a factor, designated transforming growth-factor beta, has been purified to homogeneity and molecularly cloned.[5-7,27] This factor stimulates the anchorage-independent colony growth of anchorage-dependent cells lines but has the additional property of inhibiting the growth of other cell lines in soft agar.[11,12,28] Although TGF-β can be derived from many sources, the relative abundance of this factor in platelets (estimated to be at a 100-fold greater concentration than other sources examined) suggested to us that TGF-β might be one of the factor(s) inhibiting megakaryocytopoiesis.[5]

Therefore we repeated our inhibitor studies with highly purified TGF-β. In serumless cultures, subnanomolar concentrations of this factor inhibited AchE accumulation with significant inhibition first observed at 2 pmol/L (50 pg/mL). This concentration is approximately equivalent to the amount of TGF-β in 5×10^6 platelets, based on previous data suggesting that 1 ng of the factor is releasable from 10^8 platelets.[29]

Quantitation of AchE activity in these unfractionated liquid marrow cultures does not distinguish between CFU-MK proliferation and megakaryocytic differentiation.[15] Although marrow can be separated by physical techniques into megakaryocyte-rich or CFU-MK-rich fractions, addition of TGF-β to such separated marrow would not rule out the possibility that the factor was acting indirectly via accessory marrow cells. Therefore to determine specifically the influence of TGF-β on one aspect of megakaryocytic differentiation, we studied the effect of the factor on isolated single megakaryocytes in serumless culture. As shown previously, IL 3 is one factor that promotes megakaryocytic differentiation as assessed by increments in cell size, ploidy, and AchE content.[17] At the tested concentration of 20 pmol/L, the percentage of cells expected to increase in size in response to IL 3 was markedly inhibited by TGF-β.

The inability to isolate single CFU-MK precludes a similar analysis of the influence of TGF-β on megakaryocytic progenitor cell proliferation. Nevertheless, picomolar concentrations of TGF-β inhibited both megakaryocytic and granulocyte-macrophage colony formation in a standard agar culture system. Preliminary data from Solberg et al[30] suggest that TGF-β inhibits early erythroid and multilineage colony formation in addition to megakaryocytic colony formation in man. TGF-β may thus be a general inhibitor of hematopoiesis, although its effects may not be directly on the progenitor cells themselves.

The data show that TGF-β is a potent in vitro inhibitor of murine megakaryocytopoiesis. Moreover, TGF-β is an inhibitor of the granulocytic lineage, at least at the level of the colony-forming cell. These data do not show, however, that TGF-β is the only platelet-derived inhibitor. Other factors that inhibit megakaryocytopoiesis may be present in platelet extracts, perhaps with greater lineage specificity, and further investigation of this possibility will be required. Thus the overall importance of TGF-β in the suppression of hematopoiesis in general and megakaryocytopoiesis in particular remains unclear.

Studies of the influence of TGF-β on in vivo megakaryocytopoiesis will be of interest. Hypertransfusion of platelets into experimental animals has been shown by a number of investigators to result in a decrease in the size, ploidy, and number of megakaryocytes, an effect attributed to suppression of a thrombopoietic hormone.[31] Krizsa et al have reported that administration of freeze-thawed platelet extracts also inhibits megakaryocytopoiesis.[32] It is conceivable that these in vivo effects of platelets or platelet products may not be related to suppression of a hormone but rather mediated via platelet-derived inhibitor(s), perhaps including TGF-β.

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Fetal Hemoglobin Levels and β^S Globin Haplotypes in an Indian Population With Sickle Cell Disease

By A.E. Kulozik, B.C. Kar, R.K. Satapathy, B.E. Serjeant, G.R. Serjeant, and D.J. Weatherall

To further explore the cause for variation in hemoglobin F (Hb F) levels in sickle cell disease, the β globin restriction-fragment length polymorphism haplotypes were determined in a total of 303 (126 SS, 141 AS, 17 Sβ°, 7 Aβ,$^\circ$ and 12 AA) Indians from the state of Orissa. The β^s globin gene was found to be linked almost exclusively to a β^S haplotype (+ + + − + + −), which is also common in Saudi Arabian patients from the Eastern Province (referred to as the Asian β^s haplotype). By contrast, the majority of β^A and β° thalassemia globin genes are linked to haplotypes common in all European and Asian populations (+ − − − − −[+/−]; − − + + − + +). Family studies showed that there is a genetic factor elevating Hb F levels dominantly in homozygotes (SS). This factor appears to be related to the Asian β^S globin haplotype, and a mechanism for its action is discussed. There is also a high prevalence of an independent Swiss type hereditary persistence of fetal hemoglobin (HPFH) determinant active in both the sickle cell trait and in sickle cell disease.

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THE clinical course of homozygous sickle cell (SS) disease varies from death in infancy to absence of symptoms with almost normal life expectancy.[1] An important determinant of clinical severity is the level of fetal hemoglobin (Hb F) that inhibits intracellular polymerization of sickle hemoglobin (Hb S), and hence sickling, and reduces the severity of hemolysis and the occlusion of the microcirculation. A recent study has shown that SS disease in Orissa State, India has a mild clinical picture associated with high levels of Hb F and a high prevalence of α thalassemia.[2] Classical and molecular genetic analyses in this population have produced evidence for a dominant factor linked to the β^s globin gene that is responsible for increased γ-globin gene expression in both SS disease and sickle cell β° (Sβ°) thalassemia. A separate Swiss type HPFH,[3] active in both heterozygotes (AS) and homozygotes (SS), appears to be common in these families.

PATIENTS AND METHODS

During a three-week period in February/March 1986, clinical and hematologic examinations were performed on 148 patients with sickle cell disease (131 SS; 17 Sβ° thalassemia). In addition, 142 persons with the sickle cell trait, seven with the β° thalassemia trait and 12 persons with a normal globin genotype who were parents or siblings of the index cases, were also studied. Relatives below the age of 3 years were excluded because of the effect of young age on Hb F. Peripheral blood samples were kept frozen at −20°C and within 3 weeks were transported to Oxford where $^G\gamma$ to $^A\gamma$ globin chain ratios were measured with high pressure liquid chromatography[4] and where DNA was extracted. Further aliquots of the peripheral blood samples were kept at 5°C to 10°C and were taken to Jamaica where Hb F levels were measured by alkali denaturation,[5] for which the upper limit of normal for that laboratory was 0.7%. DNA was digested with restriction endonucleases Hind II, Hind III, Xmn I, and Bam HI under conditions recommended by the manufacturers. The β globin gene haplotype was determined by Southern blot analysis[6] using the following genomic DNA fragment probes: 1.3 kb Bam HI/EcoRI ϵ; 3.3 kb Hind III γ; 1.8 kb Bgl II/Xba I $\psi\beta$; Bam HI/EcoRI IVS-2 β. In cases with two different haplotypes the correct haplotype pair was derived by family studies, but in one patient with SS disease and in eight with Sβ° thalassemia this was not possible, and it was assumed that a common haplotype was present with a rare haplotype rather than two rare haplotyes.[7]

RESULTS

Some clinical and hematologic characteristics of the SS patients are reported elsewhere.[2]

Hemoglobin F levels. The levels of fetal hemoglobin in Indian SS patients are markedly higher than in Jamaican patients.[2] Mean Hb F levels did not differ significantly between the age groups of 2 to 5 years (17.1%), 6 to 10 years (17.7%), and 11 to 20 years (17.5%) but were significantly lower in those above 20 years (14.5%). Hb F levels in Indian patients with Sβ° thalassemia were markedly higher (mean 14.9%) than in a comparable Jamaican group[1] but did not differ significantly ($P > 0.1$) from Indian SS patients. $^G\gamma/^A\gamma$ ratios in ten SS patients with Hb F levels of about 20% ranged from 0.68 to 0.82; three SS patients with Hb F levels below 10% showed ratios of 0.82, 0.84, and 0.85; and in seven other such patients ratios were above 0.6, although in these a more precise quantitation was not possible.

The mean Hb F level in 108 AS adults (20 years and above) was 0.7% and in 33 nonadults (3 to 19 years) was 0.9%. Hb F levels exceeded 1% in 25 (23%) AS adults and in nine (27%) AS nonadults. Comparison of the Hb F distribution in Indian and Jamaican AS parents with SS offspring (Fig 1) indicated that high levels occur more frequently in Indian parents. The mean Hb F level in 12 AA relatives was 0.3% (range 0.1% to 0.6%).

Family studies. Hb F levels in Jamaican patients with SS disease are known to be related to the Hb F levels in their

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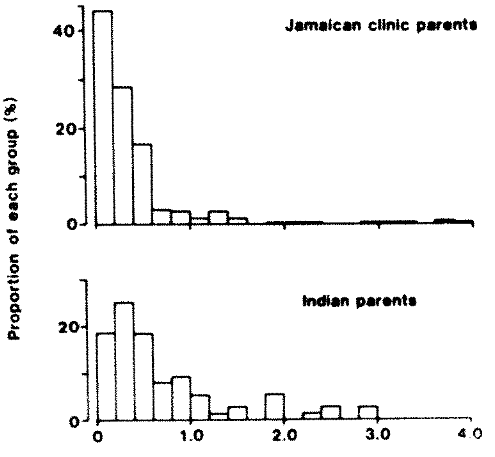


Fig 1. Comparison of Hb F levels in Indian and Jamaican AS parents of offspring with SS disease.

AS parents, rising with increasing maximum parental Hb F level.[8] Examination of Indian data reveals a similar relationship, although for any individual level of parental Hb F the Hb F levels in SS offspring are higher compared to Jamaicans (Fig 2). In 21 families in which both parents had Hb F levels below 1%, Hb F levels in the 27 offspring with SS disease (mean [SD], range; 15.4% [4.7%], 7.5% to 24.7%) were significantly lower ($P < 0.05$) than in 30 offspring with SS disease (18.8% [4.5%], 9.7% to 27.4%) among 21 families in which at least one parent had an Hb F level exceeding 1%. When Hb F levels were examined in the AS and AA offspring of such families, Hb F levels were below 1% in all 20 offspring (17 AS, 3AA) of the former type of family but exceeded 1% in 5/10 AS and in neither of the 2 AA offspring in the second type of family. Thus about 20% of the heterozygous AS offspring have a phenotype similar to the Swiss type HPFH with Hb F levels of more than 1%. This phenotype seems to segregate in a Mendelian fashion but independently of the β haplotype.

Haplotype analysis. With the combination of enzymes and probes used in the present study, the commonest β^s haplotype was + + + − + + − (Hind II ϵ, Xmn I γ, Hind III $^G\gamma$, Hind III $^A\gamma$, Hind II $\psi\beta$, Hind II 3′ $\psi\beta$, Bam HI β) and closely resembles that designated No. 31 by Antonarakis et al.[9] Since different restriction sites were used and since the haplotype is commonly linked to the β^s globin gene in Saudi Arabian[10-14] and Indian[10,14] but not in African populations, it is referred to as the Asian β^s haplotype.

In 126 SS patients analysis of the β^s haplotypes revealed that 107 (85%) were homozygous for the Asian β^s haplotype, 17 (13%) were heterozygous, and two patients had a haplotype that differed only at the Hind II ϵ site. The distribution of Hb F levels did not differ significantly between these groups (Table 1). Among 141 AS individuals the + + + − + + − haplotype occured homozygously in seven, heterozygously in 120, and did not occur on either chromosome in 14 persons (Fig 3). Mean Hb F levels in these groups expressed as the geometric means were 1.49%, 0.63%, and 0.41% respectively; analysis of variance indicated a highly significant trend ($P = 0.0002$) between groups. Hb F levels exceeded 1% in five (71%) homozygotes, in 27 (23%) heterozygotes, and in two (14%) individuals without this haplotype.

The frequency of β^S, β^A, and β^o thalassemia haplotypes in unrelated individuals (Table 2) indicates that the β^s mutation occurs predominantly on the Asian haplotype and β^o thalassemia mutations on the + − − − − − + haplotype. Normal β globin genes were carried on chromosomes characterized by a variety of different haplotypes, most frequently the + − − − − − + or the − − + + − + + pattern.

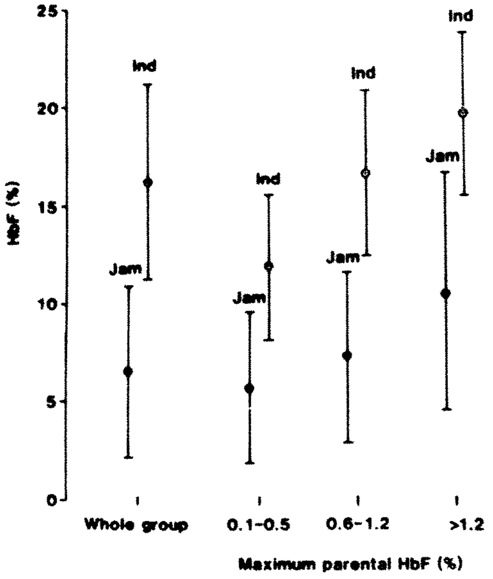
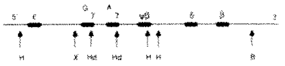


Fig 2. Distribution of Hb F levels (mean ± 1 SD) in Indian and Jamaican patients with SS disease according to maximal parental Hb F levels.

DISCUSSION

Some of the clinical variability of SS disease has been attributed to raised Hb F levels causing a milder clinical

Table 1. β Globin Haplotypes, Hb F Levels, and Ages of Indian Patients with SS Disease and $S\beta^o$ Thalassemia

Globin Genotype	Haplotype 	Number	Mean Hb F (%)	Mean Age (Years)
SS	+ + + − + + −/+ + + − + + −	107	16.4	16.6
	+ + + − + + −/− + + − + + −	15	15.5	13.3
	+ + + − + + −/+ − − − − − +	1	19.3	4
	+ + + − + + −/+ − − − − − −	1	22.3	4
	− + + − + + −/− + + − + + −	1	24.5	18
	− + + − + + −/+ − − − − − −	1	10.5	8
$S\beta^o$	+ + + − + + −/+ − − − − − +	15	14.3	20.2
	+ + + − + + −/− − + + − + +	1	18.4	24
	+ + + − + + −/− + + − + + −	1	18.7	13

The map represents the β globin gene cluster with restriction endonuclease dimorphisms used in this study.

Abbreviations: H, Hind II; Hd, Hind III; B, Bam HI; X, Xmn I; +, presence of the respective restriction site; −, absence of the respective restriction site.

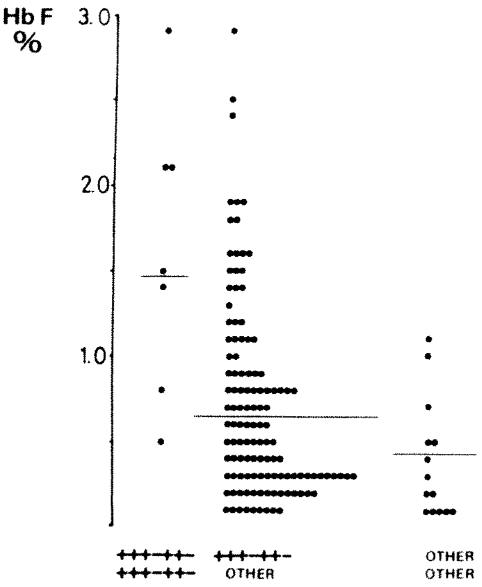


Fig 3. Hb F levels of heterozygous (AS) relatives of SS disease patients according to their haplotype combination.

picture.[1] Elevated Hb F levels with a mild clinical phenotype appear common in Arab and Iranian patients.[15-19] The limited data previously available suggested that elevated Hb F levels were also common in Indian patients.[20-22] Selected family studies have suggested that the high Hb F levels might be due to an HPFH determinant linked to the β^s globin gene[8,10,23,24] and associated with a particular β^s haplotype (+ + + − + + −).[10-13] Furthermore, the geographic distribution of this haplotype coincides with the occurrence of high Hb F levels and of mild SS disease.[14]

In the present study of a large number of Indian patients with SS disease and Sβ° thalassemia, the Hb F level was found to be raised and the β^s globin gene was almost exclusively linked to the Asian β^s haplotype. The basis of this relationship is unclear. Among AS individuals there was evidence that Hb F levels were directly related to the number of copies of the Asian β^s haplotype inherited, consistent with a genetic determinant linked to this haplotype. However, the SS population was so homogeneous (85% being homozygous for the Asian β^s haplotype) that it was unsuitable for examining whether this haplotype was linked to factors determining Hb F level. Indeed, only two patients did not possess this haplotype, but in both there was a closely related haplotype. It is conceivable that in this population the Hb F levels observed in SS disease are not linked to the Asian β^s haplotype but reflect some other genetic factor common in Asian Indians that facilitates increased γ-globin gene expression under erythropoietic stress. However, most patients with homozygous β thalassemia of mixed Asian Indian origin are transfusion dependent with low Hb F levels,[25] and the genetic background is similar to Orissan Indians as the common β^A haplotypes in these two populations are identical (Table 2).[26] It is of interest that patients with homozygous β thalassemia with a haplotype closely related to the Asian β^s haplotype have higher Hb F levels[25] and that this observation together with the data on the Asian β^s haplotype in this report support the hypothesis that a determinant for raised Hb F levels is linked to that haplotype. Furthermore, the HPFH factor linked to the Asian β^S haplotype seems to be dominant in SS disease[10,11] and Sβ° thalassemia (Table 1), as one copy of the Asian β^s haplotype is sufficient to raise the Hb F level. The mechanism for this effect is unclear. One possibility is a gene closely linked to the Asian haplotype that encodes for a factor acting in trans on both chromosomes. Alternatively, a site, such as a promoter region, within the Asian haplotype might be capable of locally concentrating transacting factors that might then influence the paired homologous allele. This latter mechanism has been suggested in drosophila where transcriptional enhancement of the Sgs-4 gene was shown to be mediated by diffusible factors that are at a high local concentration and can be utilized by paired homologous genes within the same domain.[27]

Table 2. Haplotype Frequencies of Independent β^s, β°, and β^A Chromosomes in Orissan Indians

Haplotype	β^s	β°	β^A
+ + + − + + −	187 (89.9%)	— — —	5 (4.2%)
− + + − + + −	18 (8.6%)	1 (8.3%)	7 (5.9%)
− + + − + + +	—	— — —	13 (10.9%)
− − + + − + +	—	1 (8.3%)	23 (19.3%)
+ − − − − − +	1 (0.5%)	10 (83.4%)	56 (47.1%)
+ − − − − − −	2 (1.0%)	—	8 (6.7%)
Others	—	—	7 (5.9%)
Totals	208	12	119

The map is as in Table 1. Other haplotypes (+ − − − − + −; + − − − + + −; − − − − − − +; − − + + − + −; + + + − + + +; − − − − + + +; + + + + + + +) were observed on one chromosome only.

It has been shown previously that a C→T mutation at position −158 5′ to the $^G\gamma$ globin gene that introduces an Xmn I restriction site is linked to the core haplotype + − + + (Hind IIIγ, Hind III$\psi\beta$) and that it is associated with a raised $^G\gamma/^A\gamma$ globin chain ratio.[28-31] This site is present in the common Indian β^S haplotype, confirming the tight linkage of the $^G\gamma$ − 158 C→T mutation to the + − + + core haplotype, and our findings also show its association with raised $^G\gamma/^A\gamma$ globin chain ratios. The lack of SS patients not carrying the $^G\gamma$ − 158 C→T mutation in this study—only three are heterozygous and all the others homozygous for this mutation—unfortunately does not allow us to address the question whether the $^G\gamma$ −158 C→T mutation causes raised Hb F levels in Indian patients with SS disease.

Whatever mechanism is involved, inheritance of the Asian β^s haplotype is not the only determinant of Hb F levels in this population. In some families there appeared to be an HPFH determinant, phenotypically of the Swiss type,[3] which segregated independently of the Asian β^s haplotype. Thus raised Hb F levels in AS offspring segregated in families where at least one parent had a high Hb F level and even within the SS population who were mostly homozygous for the Asian β^s haplotype, the mean Hb F differed by a statistically significant 3%, depending on whether an AS parent manifested a raised Hb F level. There appear, therefore, to be at least two determinants of the Hb F level in this Indian population. An HPFH determinant that has also been recognized in Jamaicans accounts for the increasing Hb F levels observed with increasing parental Hb F levels. However, for each level of parental Hb F, the Hb F observed in Indian SS offspring exceeds that in comparable Jamaicans, and this difference may be related to possession of the Asian β^s haplotype.

Of the three β^s globin haplotypes recognized in homozygous sickle cell disease in Africa, only one, designated the Senegal haplotype, was associated with high Hb F levels.[28] The close similarity between the Senegal haplotype $(-\underline{++-++}+)$ and the Asian β^s haplotype $(+\underline{++-++}-)$ is therefore of interest. The characterization of factors responsible for raised Hb F levels is clearly important in understanding the natural history of sickle cell disease, is of clinical prognostic interest, and is likely to shed some light on mechanisms of globin gene regulation. The Asian β^S haplotype $(+++-++-)$ seems to be linked to such an HPFH determinant; confirmation of this would require an Asian population with greater heterogeneity of β^s haplotypes, allowing study of the relationship of Hb F to different combinations.

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Myeloablation With Diaziquone: In Vitro Assessment

By Brian H. Kushner, Salvatore Siena, and Hugo Castro-Malaspina

The promising antineoplastic agent diaziquone is associated with prolonged aplasia and rare instances of bone marrow necrosis, but only mild extramedullary toxicity. To explore the drug's potential as a myeloablative agent prior to bone marrow transplantation, we compared its effects on hematopoietic versus marrow stromal cells. After short-term (one to six hours) or prolonged (three to seven days) exposure to the drug, marrow was assayed for hematopoietic (CFU-Mix, BFU-E, CFU-GM) and stromal (CFU-F) colony-forming cells and studied in long-term marrow culture (LTMC). One- and three-hour treatments produced little cytotoxicity, even at 5000 ng/mL. After six-hour treatments with this dose, marrow was depleted of CFU-Mix, BFU-E, and CFU-GM, but produced CFU-GM in LTMCs, indicating an ongoing input of CFU-GM from a surviving pre-CFU-Mix population. In contrast, elimination of the latter may be inferred from the absence of CFU-GM in LTMCs exposed for three to seven days to diaziquone at only 150 ng/mL. Under these conditions, CFU-F recovery was 40% and adherent stromal layers in LTMCs were similar to untreated controls regarding rate of development and cellular composition. Our in vitro pre-CFU-Mix-ablative regimen correlates with clinical data that show prolonged but reversible myelosuppression at steady-state diaziquone plasma levels of 101 ± 10 ng/mL (mean ± standard error of mean) during 7-day constant infusions. In conclusion: (*a*) hematopoietic cells are more sensitive than marrow stromal cells to the dose- and highly time-dependent cytotoxicity of diaziquone, (*b*) a direct drug-induced noxious effect on the marrow microenvironment is an unlikely cause of the isolated episodes of marrow necrosis after the use of diaziquone in vivo, and (*c*) prolonged infusion of diaziquone represents an attractive means for achieving myeloablation in selected clinical situations.

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DIAZIQUONE (AZQ, NSC 182986) is a lipophilic quinone that was rationally synthesized to optimize blood-brain barrier penetration.[1] Its antineoplastic effect[2-6] has been attributed to alkylating activity.[7] Its slow uptake by leukemic cells[8] but short plasma half-life[1,3,9-11] led to phase I/II trials of prolonged infusions.[6] Pancytopenia, not extramedullary toxicity, was the dose-limiting factor. This observation suggested that diaziquone may be best exploited in the context of bone marrow transplantation. Bone marrow necrosis, however, occurred in two of three patients given maximal doses, raising concern that diaziquone may damage the hematopoietic microenvironment and thereby jeopardize engraftment.[12] We investigated this issue by using clonal assays and long term marrow cultures (LTMCs) to correlate the drug's ablative capacity on hemopoietic cells with its effects on marrow stromal cells. Our studies underscore the usefulness of LTMCs for investigating drug effects on hematopoietic and nonhematopoietic components of marrow, demonstrate that diaziquone's cellular kinetics render LTMCs particularly appropriate for defining the effects on human marrow of prolonged exposure to this drug, and support the pretransplant use of diaziquone in myeloablative doses.

MATERIALS AND METHODS

Bone Marrow Cells

Heparinized marrow aspirated from the iliac crests of healthy volunteers who gave informed, written consent were centrifuged at 200 g for ten minutes. The buffy coat cells were collected and washed. For some experiments, marrow was fractionated by agglutination with soybean lectin and by rosetting with sheep erythrocytes to isolate a target population (SBA-E–cells) enriched for hematopoietic cells and 97% to 99+%-depleted of stromal cells[13] and T cells.[14]

Diaziquone Preparation and Drug Exposure

Diaziquone was provided in 10-mg vials by the National Cancer Institute. It was reconstituted in *N,N*-dimethylacetamide and phosphate buffer saline immediately before use. Further drug dilutions were prepared with the medium appropriate for the specific culture technique being used. When reconstituted in solution maintained at pH 7.0, diaziquone has a half-life of 17 days.[15]

Marrow buffy coat cells in alpha-medium (Flow Laboratories, Hamden, CT) with 10% heat-inactivated fetal calf serum (FCS) were incubated at 5×10^6 cells/mL with graded doses of diaziquone for 1, 3 or 6 hours at 37°C in a humidified 5% CO_2 atmosphere, and washed three times prior to use. In experiments examining the effects of prolonged drug exposure, marrow cells were resuspended and cultured in medium containing diaziquone in the amounts needed to achieve the desired drug concentration.

Culture Techniques

Multilineage and erythroid colony-forming cells. Mononuclear cells were recovered from untreated or diaziquone-treated marrow buffy coat cells by neutral density centrifugation in an isotonic sterile Percoll solution, 1.074 g/mL, 270 mosm (Pharmacia, Piscataway, NJ). These cells or SBA-E–cells were cultured[16] in quadruplicate in 35-mm dishes (Lux Scientific Co, Newburg, CA) at 1.0×10^5/mL of 1% methylcellulose in Iscove's modified Dulbecco medium with 30% human plasma, 10% phytohemagglutinin-stimulated leukocyte-conditioned medium, 5×10^{-5} mol/L 2-mercaptoethanol, and one unit of human recombinant erythropoietin (AMG-

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en, Thousand Oaks, CA). CFU-Mix and BFU-E were scored on day 14.

Granulocyte-macrophage colony-forming cells. Marrow cells were cultured as described[17] in quadruplicate in 35-mm dishes (Corning Glass Works, Corning, NY) at 2 × 10^5/mL of 0.3% agar in supplemented McCoy's 5A medium and 10% FCS. In each dish, 0.1 mL of medium conditioned by the 5637 bladder carcinoma cell line stimulated colony formation.[18] CFU-GM (more than 50 cells/aggregate) were scored on day 7.

Fibroblast colony-forming cells. Marrow buffy coat cells (5 × 10^6) in 20 mL alpha-medium with 20% FCS were set up in T-75 flasks (Corning) that were gassed with 5% CO_2 in air and kept at 37°C. Medium was changed on day 4 in cultures of cells preincubated with diaziquone for 1, 3, or 6 hours. In cultures exposing cells to diaziquone for three or seven days, the initial medium contained graded amounts of diaziquone but was replaced by drug-free medium on day 3 in one set of experiments, and on day 7 in another set. Cultures were stopped on day 10, stained with Wright-Giemsa solution, and scored for CFU-F (more than 50 cells/aggregate).[19]

Long-term marrow cultures. Marrow buffy coat cells (20 × 10^6) suspended in 10 mL medium were inoculated into T-25 flasks (Corning) that were gassed with 5% CO_2 in air, and kept at 37°C. The medium consisted of supplemented McCoy's 5A medium,[17] 12.5% horse serum, 12.5% FCS, and 10^{-6} mol/L hydrocortisone sodium succinate, with or without graded amounts of diaziquone, depending on the experimental design. On day 3 in one set of experiments and on day 7 in a separate set, supernatants were purged of erythrocytes and mature myeloid cells by Percoll separation; the mononuclear cells were returned to their flasks in fresh, drug-free medium. LTMCs were then maintained at 33°C. Half of the supernatant was removed weekly and the cells in suspension were assayed for CFU-GM. At week 3, adherent layers in some flasks were suspended[17] using a nontoxic 0.01% (wt/vol) Ca^{++}- and Mg^{++}-free trypsin (GIBCO, Grand Island, NY) solution to determine their CFU-Mix, BFU-E, and CFU-GM content.

To monitor stromal layer development, the extent of flask coverage by an interlocking network of cells was estimated weekly by phase microscopy. Cellular composition was assessed in situ by immunofluorescence methods.[17] Fibroblasts were identified by rabbit antibodies to fibronectin (Bethesda Research Lab, Gaithersburg, MD) and to collagen type III (Dr H Furthmayr, Yale University). Endothelial cells and macrophages were identified by mouse monoclonal antibodies (Australian Monoclonal Development, Antarmon, Australia) to factor VIII-related protein and to human monocytes/macrophages, respectively. Adipocytes were distinguished by their morphology.

Statistical Analysis

With the Minitab package program (Duxbury Press, Boston), regression analyses were performed for the clonogenic assays using the log of the percent of the control as the dependent variable and the diaziquone dose as the independent variable. Lines were forced through the value of the log of 100% at dose zero. Slopes of the fitted lines were compared based on F-tests computed from residual sums of squares.

RESULTS

Effect of Diaziquone on Marrow Colony-Forming Cells

Diaziquone exerted dose- and time-dependent killing of clonogenic hematopoietic and stromal cells (Fig 1). One-hour treatments did not significantly affect recovery of these cells, even at high drug concentrations (500 to 5,000 ng/mL, data not shown). With longer treatments, increasing doses caused an exponential decrease in progenitor recovery. Three-hour treatments with 1,000 ng/mL produced approximately 30% reductions in CFU-Mix, BFU-E, and CFU-GM survival, and 10% to 15% decrease in CFU-F. With 6-hour treatments, doses that inhibited 50% (ID_{50}) of CFU-Mix, BFU-E, and CFU-GM were 160 ng/mL, 200 ng/mL, and 245 ng/mL, respectively, which were significantly lower than the ID_{50} of 530 ng/mL for CFU-F ($P = .001$). CFU-Mix and BFU-E did not differ significantly in sensitivity to diaziquone ($P = .15$), but these progenitors were significantly more sensitive than CFU-GM ($P = .005$). After 6-hour exposure to 5,000 ng/mL, hematopoietic progenitors were not detected, but CFU-F recovery was 10%.

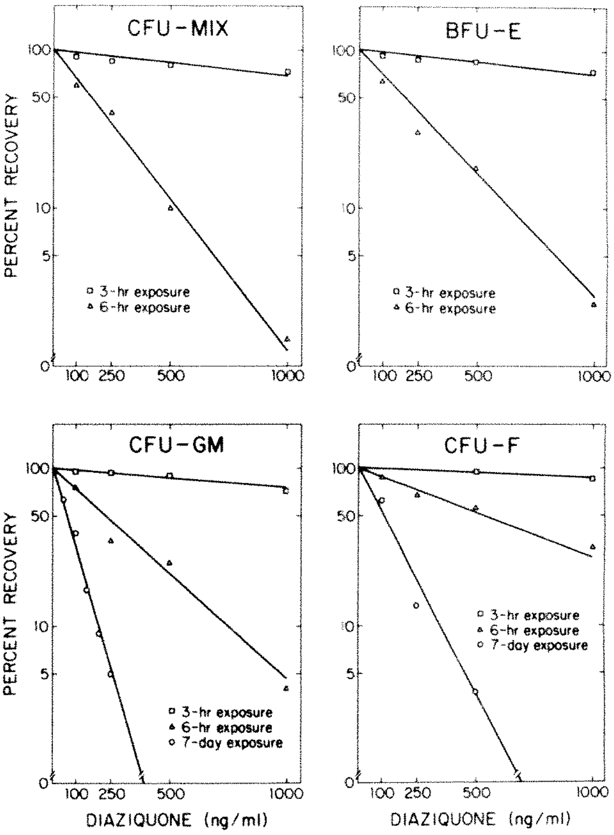

Fig 1. Survival curves of marrow hematopoietic and stromal progenitors. Results shown are the mean (SEM less than ±10%) of three (CFU-Mix, BFU-E) and four (CFU-GM, CFU-F) experiments at each exposure time. Controls were: CFU-Mix, 19 ± 2 per 4 × 10^5 cells; BFU-E, 94 ± 9 per 2 × 10^5 cells; CFU-GM, 91 ± 7 per 10^5 cells; and CFU-F, 131 ± 15 per 5 × 10^6 cells. Note the marked time-dependency of diaziquone cytotoxicity on all four cell types.

When marrow buffy coat cells were plated with graded amounts of diaziquone in the culture medium, ie, 7-day constant exposure, the ID_{50} of CFU-GM formation was 60 ng/mL, and CFU-GM recovery was 5% at 250 ng/mL (Fig 1). To ascertain whether this inhibition was a direct effect on hematopoietic cells or was mediated via effects on accessory "helper" cells, SBA-E-cells were cultured under the same constant-exposure conditions. Survival curves of CFU-GM matched those observed using buffy coat cells (data not shown).

When marrow buffy coat cells were cultured for CFU-F with graded amounts of diaziquone in the medium for seven days, ie, 7-day constant exposure, the ID_{50} of CFU–F was 110 ng/mL (Fig 1). Similar results—CFU–F ID_{50} of 145 ng/mL—were obtained with 3-day constant exposures (data not shown).

Effect of Diaziquone on LTMCs

Diaziquone dosage and length of treatment significantly affected LTMCs regarding CFU–GM production, the rate of development, and the cellular composition of adherent layers. Marrow suspensions treated for one or three hours (Fig 2) with doses of diaziquone up to 5,000 ng/mL and then set up in LTMCs produced CFU–GM for five to six weeks and gave rise to adherent stromal layers that, similar to controls, reached confluence within three to four weeks and contained fibroblasts, macrophages, adipocytes, and rare endothelial cells (Table 1).

Treatments for six hours (Fig 3) caused more marked dose-dependent reductions in CFU–GM yields. In particular, following 6-hour treatments with very high doses of diaziquone, eg, 5,000 ng/mL, marrow suspensions that were depleted of CFU–Mix, BFU–E, and CFU–GM, as measured by direct assay in semisolid medium, generated CFU–GM for five weeks in LTMCs. Six-hour treatments with diaziquone up to 500 ng/mL did not adversely affect stromal layer development or composition (Table 1) as compared to controls; at 1,000 ng/mL, however, confluence was very delayed, and at 5,000 ng/mL confluence was not attained.

LTMCs exposed to diaziquone for three or seven days (Fig 4) at doses up to 100 ng/mL generated CFU–GM for five or more weeks. Doses of 150 ng/mL or greater abolished recovery of CFU–GM from either the nonadherent or the 3-week-old adherent cellular compartments of LTMCs. Prolonged exposure to doses of diaziquone up to 250 ng/mL retarded the development of confluent stromal layers but did not alter the heterogeneous cellular composition as compared to untreated controls (Table 1). After prolonged exposures to 500 ng/mL, confluence did not develop; only fibroblasts and adipocytes were identified. Higher doses resulted in occasional-to-rare fibroblastic areas.

DISCUSSION

Our studies demonstrate differential sensitivities to diaziquone of hematopoietic as compared to marrow stromal cells, highlight the time-dependency of diaziquone's cytotoxic activity, and support using prolonged, myeloablative infusions of diaziquone in conjunction with bone marrow transplantation.

One- and three-hour treatments with up to 5,000 ng/mL diaziquone had little effect on hematopoietic progenitors, whereas 7-day exposure to doses as low as 50 ng/mL had a significant cytotoxic impact on these cells. Data from CFU–Mix, BFU–E, and CFU–GM, however, may not apply to the pluripotent stem cell that constitutes the target of definitive myeloablative procedures prior to bone marrrow transplantation. Differences in drug effects on committed versus pluripotent hematopoietic cells are inferred from the in vivo hematologic regenerative capacity of harvested marrow depleted of CFU–Mix, BFU–E, and CFU–GM by purging with 4-hydroperoxycyclophosphamide (4-HC).[20,21] Direct measurement of the pluripotent stem cell is not now feasible in humans, but LTMCs may provide an indirect means for detecting hematopoietic cells that are more primitive than CFU–Mix. We observed sustained production of CFU–GM in LTMCs initiated with marrow completely depleted of CFU–Mix, BFU–E, and CFU–GM by 6-hour treatments with 5,000 ng/mL diaziquone. These findings, similar to results with anti-Ia antibody,[22] 4-HC,[23] and VP-16,[24,25] indicate an ongoing input of CFU–GM from a surviving, pre-CFU–Mix population. In sharp contrast, CFU–GM were not recovered from LTMCs following three- or seven-day exposures to 150 ng/mL diaziquone. These data support an ablative effect on pre-CFU–Mix of prolonged exposure to low doses of this drug.

Although eliminated from LTMCs exposed for three to seven days to 150 ng/mL diaziquone, CFU–GM are detectable at seven days when marrow is cultured in semisolid medium containing doses up to 250 ng/mL. These seemingly inconsistent findings are not attributable to diaziquone

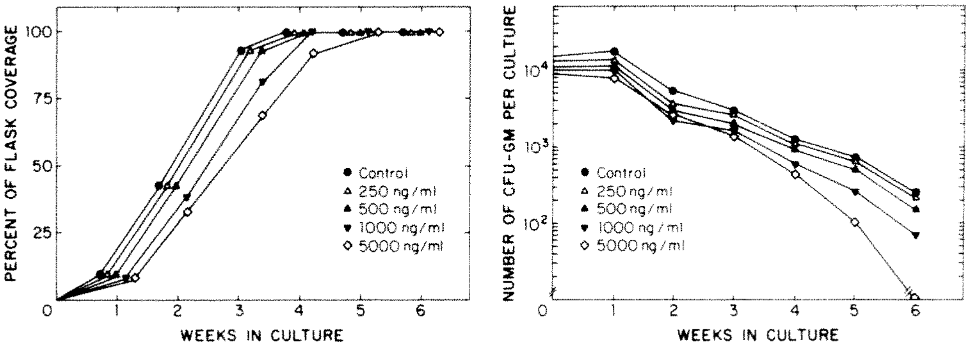

Fig 2. Effects of 3-hour treatments with diaziquone on LTMC stromal layer development (left) and CFU–GM generation (right). Note the relatively minor differences in these parameters over a wide range of diaziquone dosages. Results shown are the mean of three experiments.

Table 1. Composition of Stromal Layers in LTMCs Derived From Diaziquone-Treated Bone Marrow

Length of Exposure	250 ng/mL				500 ng/mL				1,000 ng/mL				5,000 ng/mL			
	F	M	Ad	EC	F	M	Ad	EC	F	M	Ad	EC	F	M	Ad	EC
3 hr	4+	2+	3+	1+	4+	2+	3+	1+	4+	2+	3+	1+	3-4+	1-2+	2+	±
6 hr	4+	2+	3+	1+	4+	2+	3+	1+	4+	1+	1-2+	0	2-3+	0	1+	0
3 days	4+	2+	3+	0-1+	2-3+	0	1-2+	0	1+	0	0	0	0-1+	0	0	0

The presence and relative amount of different types of adherent stromal cells was semiquantitatively assessed. Control stromal layers derived from 20×10^6 untreated marrow buffy coat cells exhibited the following composition: F, 4+; M, 2+; A, 3+; EC, ± – 1+.

F, fibroblast; M, macrophage; Ad, adipocyte; EC, endothelial cell.

effects on accessory cells, since CFU-GM survival curves using buffy-coat cells or stromal-cell- and T-cell-depleted SBA-E-marrow fractions overlap. Diaziquone's cellular kinetics provide a more convincing explanation. Slow cellular uptake[18] rather than cell-cycle-specificity[26] accounts for the time-dependency of diaziquone's cytotoxicity. When marrow is assayed for CFU-GM by direct plating in semisolid medium containing diaziquone (ie, constant 7-day drug exposure) progeny—appearing as colonies of cells—are generated before sufficient drug is accumulated to produce a cytotoxic effect on the progenitor cell. In contrast, when the effects of prolonged drug exposure are studied using LTMCs, drug uptake proceeds for several days, reaching cytotoxic intracellular levels, before hematopoietic progenitor cell assays are performed. In assessing the cytotoxicity of prolonged diaziquone exposure, therefore, we view the LTMC data as more reflective of the in vivo situation than the data from semisolid medium assays. In fact, the hematopoietic-stem-cell ablative diaziquone regimen defined in our system—3-day exposure to 150 ng/mL—correlates with clinical experience[6]: prolonged but reversible myelosuppression followed seven-day constant infusions of diaziquone that produced steady-state plasma concentrations of 101 ± 10 ng/mL (mean ± SEM; range 54-150 ng/ml). Presumably, higher drug levels (as in our system) cause myelosuppression that is unacceptable in the absence of bone marrow rescue.

Bone marrow necrosis in two of three patients treated by prolonged infusions of maximal doses of diaziquone[6] raises the issue of drug-induced damage to the marrow microenvironments.[12] Such an effect might block hematologic recovery posttransplantation of bone marrow and would make this method of diaziquone adminstration unsuitable for pretransplant use. Our in vitro hematopoietic-stem-cell ablative diaziquone regimen is relatively sparing of marrow stromal progenitors, as shown by the 40% recovery of CFU-F. Moreover, adherent layers that formed in LTMCs after prolonged treatments with up to 250 ng/mL of diaziquone attained confluence and resembled untreated controls regarding heterogeneity of cellular composition. We interpret these data as supporting the analysis[6] that other factors, eg, vessel occlusion secondary to rapid and extensive cell lysis within a markedly hypercellular marrow,[27] caused the necrosis. Additional support for this view comes from radiation data. In vitro radiation at 480 to 840 cGy (depending on the dose rate) abolishes human CFU-F recovery and inhibits LTMC stromal layer development,[28,29] yet patients irradiated with up to 1,440 cGy routinely engraft with bone marrow transplants,[30] indicating an intact marrow microenvironment. It may be deduced that since (relative to their respective myeloablative dosages) diaziquone is much less toxic in vitro than radiation to marrow stromal cells, a similar situation prevails in vivo.

Total body irradiation and busulfan, the conventional methods of myeloablation prior to bone marrow transplantation, have overlapping pulmonary toxicities[31] that make it risky to use both modalities in the same patient within a short period of time. Yet, repeat or intensified myeloablative measures may be indicated (*a*) to prepare patients for a second transplant,[32,33] (*b*) to treat refractory acute leukemias, or (*c*) to ablate the expanded, abnormal primitive-stem-cell compartments of disorders such as juvenile chronic myeloid leukemia (JCML) or blastic-phase chronic myelogenous leukemia (Ph'+ CML).[34-36] Intensive diaziquone regimens—25 to 35 mg/m^2/d × 5 to 7 days by bolus

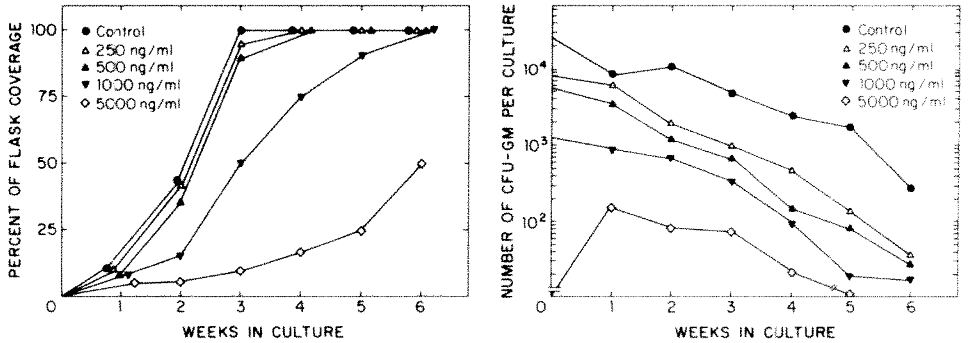

Fig 3. Effects of 6-hour treatments with diaziquone on LTMC stromal layer development (left) and CFU-GM generation (right). Results shown are the mean of three experiments. Note the prolonged generation of CFU-GM in LTMCs derived from marrow suspensions that at the time of LTMC initiation were depleted by treatments with 5,000 ng/mL of directly measurable hematopoietic progenitors.

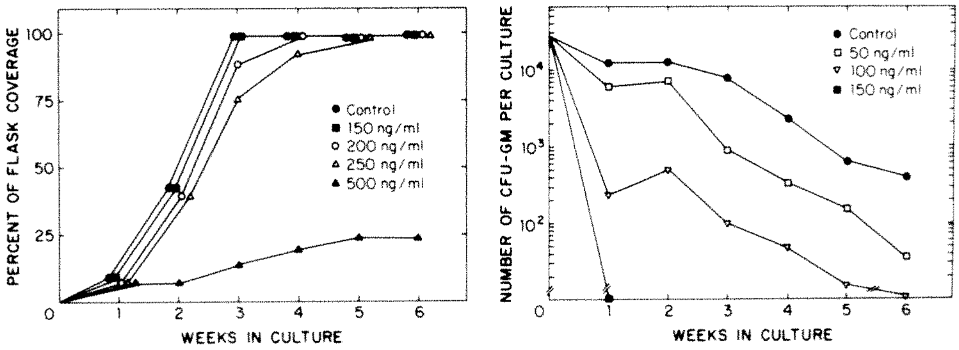

Fig 4. Effects of 3-day exposures to diaziquone on LTMC stromal layer development (left) and CFU-GM generation (right). Results shown are the mean of four experiments and are similar to those obtained with 7-day treatments. Note that prolonged treatment with 150 ng/mL abolishes CFU-GM generation but does not impair stromal layer development.

injections[4,5] or by constant infusion[6]—are effective in clearing blasts from patients with refractory or relapsed acute nonlymphocytic leukemias and from patients with panmyelopathies such as JCML, Ph'+ CML, and secondary leukemia. These results have been clouded, however, by prolonged, life-threatening aplasia. No significant extramedullary toxicity, pulmonary in particular, has been seen at doses used thus far.[6,37] Our data indicate that a diaziquone regimen adjusted to maintain plasma levels of approximately 150 ng/mL for three to seven days is myeloablative without undue toxicity to the marrow microenvironment. Taken together, these clinical and laboratory findings favor incorporation into transplant preparative protocols of this relatively nontoxic approach when, as in the clinical situations listed above, repeat or highly aggressive myelotoxic measures are desired. Since other studies demonstrate that prolonged exposure to suprapharmacologic doses of diaziquone does not significantly suppress lymphocyte mitogenic responses significantly (Chin LJ, Kushner BH, Cunningham-Rundles S, Castro-Malaspina H: unpublished data), the use of diaziquone in the allogeneic marrow transplant setting must be coupled with immunosuppressive measures.

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Favism: Impairment of Proteolytic Systems In Red Blood Cells

By A. Morelli, M. Grasso, T. Meloni, G. Forteleoni, E. Zocchi, and A. De Flora

Red blood cells (RBC) from favic patients are characterized by (*a*) severe oxidative damage (contributed by autoxidation of divicine and isouramil, two pyrimidine aglycones present in fava beans) and (*b*) greatly increased calcium levels. In vitro, both autoxidation of divicine and calcium loading produced marked alterations of proteolytic systems in intact RBC. Specifically, autoxidizing divicine inactivated procalpain, the proenzyme species of calcium-activated cytosolic neutral proteinase, or calpain. Inactivation was much greater with glucose-6-phosphate dehydrogenase (G6PD)-deficient RBC than with normal RBC. On the other hand, loading of normal and G6PD-deficient RBC with calcium resulted in (*a*) conversion of procalpain to calpain and eventual autoproteolytic inactivation of calpain itself, and (*b*) extensive release of acid endopeptidase activity from the membranes into the cytosol. Damaged RBC from favic patients had significantly lowered procalpain activity and an abnormal subcellular distribution of acid proteinase activity that was found mostly in the cytosol. When purified calpain was incubated with membranes from acetylphenylhydrazine (APH)-treated RBC, significant proteolysis was observed affecting mostly band 3 and hemoglobin chains, ie, the two proteins involved in the onset of aggregation of Heinz bodies. Moreover, exposure of intact RBC to 20 mmol/L APH induced depletion of procalpain activity for which the time course was inversely related to formation of Heinz bodies. These findings support the role of procalpain in protecting G6PD-deficient RBC from oxidant-induced Heinz body formation and imply that exhaustion of the procalpain-calpain system is an important step in the mechanisms of RBC damage and destruction in favism.

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FAVISM RECENTLY HAS been shown to be characterized by heavily disordered red blood cell (RBC) calcium homeostasis.[1,2] The intraerythrocytic calcium levels were found to be several-fold higher and, concomitantly, calcium ATPase activity was significantly reduced in RBCs from patients experiencing acute hemolysis following ingestion of fava beans, as compared with RBCs from healthy glucose-6-phosphate dehydrogenase (G6PD)-deficient subjects. These changes were also seen upon incubation of RBCs with divicine,[3] a hydroquinonic pyrimidine aglycone that, together with its cogener isouramil, has been implicated as a cytotoxic component of fava beans.[4] Cytotoxicity of divicine and isouramil is mediated by their autoxidation, which results in the formation of both quinoid species and H_2O_2.[5]

A selective target of divicine autoxidation is procalpain, the intrinsically inactive proenzyme form of the calcium-activated neutral proteinase, or calpain.[6] In addition, intracellular elevation of calcium triggers conversion of procalpain to calpain and the eventual autoproteolytic inactivation of calpain itself in the intact RBC.[7] These experimental findings prompted us to explore the properties of the proteolytic systems of RBCs during acute hemolysis of G6PD-deficient subjects in favism. Data from this study and from studies using purified procalpain indicate that the procalpain-calpain system may represent an important defense mechanism against oxidant-induced formation of Heinz bodies. Accordingly, this proteolytic system seems to prevent, at least to some extent, the G6PD-deficient RBCs from being sequestered and removed by the spleen.

MATERIALS AND METHODS

Blood samples and biochemical assays. Blood samples from 19 male subjects were investigated in this study according to the principles of the Helsinki declaration. Of these subjects, four with normal G6PD activity had the normal wild-type enzyme (B). Another subject with G6PD B had high reticulocytosis (31%) because of a gastric hemorrhage. The other 14 subjects, all of Sardinian ancestry, were G6PD-deficient. Their levels of RBC G6PD activity, estimated by the spectrophotometric assay described previously,[8] ranged between 0.0008 and 0.009 IU/g Hb. Eight of these G6PD-deficient subjects were completely asymptomatic and represented controls. The other six G6PD-deficient subjects (three children and three adults) were patients during acute hemolysis following ingestion of fava beans, as indicated by severe anemia, massive hemoglobinuria, high amounts of Heinz bodies, reticulocyte counts above 5% (up to 60% in an adult patient), methemoglobin levels above 5%, and intraerythrocytic calcium (determined by atomic absorption spectrophotometry)[1] above 120 μmol/L.

Blood samples were drawn and defibrinated with glass beads. Leukocytes and residual platelets were removed according to the procedure of Beutler et al,[9] and RBCs were processed within 24 hours for assays of proteolytic activities. Assays of procalpain activity in 1.0-mL samples of packed RBCs were carried out following removal of the endogenous protein inhibitor by chromatography of the hemolysates on a column (0.7 × 10 cm) of diethylaminoethanol (DEAE)-cellulose.[10] Procalpain activity was assayed according to Melloni et al,[11] using acid-denatured globin as substrate in the presence of 1 mmol/L Ca^{2+}. In these conditions procalpain undergoes limited auto-proteolysis to yield the fully active calpain species. Procalpain activity was expressed as units/mL of packed RBCs and 1.0 mL of RBCs was considered to contain 330 mg hemoglobin (Hb) as measured before the chromatography step.[6] Purification of procalpain from human RBCs was carried out according to Melloni, et al.[11]

Assays of acid endopeptidase activity were performed.[7] Both membrane-bound and cytosolic activities were expressed as units/mL of packed RBCs. In the case of membrane-bound acid endo-

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peptidase activity, this figure corresponded to units/5.0 mg of membrane protein. In all the favic patients with acute hemolysis, membranes contained large amounts of cytosolic proteins, mostly denatured hemoglobin (see Results); therefore, we estimated the precise content of membrane proteins by subtracting the hemoglobin levels, assayed according to Bodemann and Passow,[12] from the total protein content. Assay of cytosolic acid endopeptidase activity was preceded by DEAE-cellulose chromatography of the relevant hemolysates.[13]

Analysis of membrane proteins was performed on SDS polyacrylamide slab gels according to Fairbanks et al[14] by running 8 μg of stromal protein on each lane. Fractionation of RBCs according to density was obtained by centrifugation on discontinuous gradients of Stractan II.[15,16] Four cell layers were obtained, defined as fractions 1, 2, 3, and 4 respectively, in order of increasing density. Fractions 2, 3, and 4 were usually pooled, yielding the "dense RBC fraction." Reticulocytes were counted on dry preparations after supravital staining; usually at least 1,000 cells were counted.

In vitro incubations of RBCs. RBCs were incubated in air at a final 10% hematocrit for 30 minutes at 37°C in 0.04 mol/L *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), pH 7.4, containing NaCl up to an osmolarity of 310 mosm/L H_2O (isoosmotic TES-NaCl), 5 mmol/L glucose and divicine at the concentrations indicated. Control samples lacking divicine were incubated in parallel. Reduced divicine was prepared fresh before each experiment by acid hydrolysis of commercial vicine (Serva, Feinbiochemia GMBH & Co., Heidelberg, FRG).[1]

RBCs were loaded with calcium upon incubation at a final 10% hematocrit for 30 minutes at 37°C in isoosmotic TES containing 5 mmol/L glucose, 5 μmol/L A 23187, and 100 μmol/L $CaCl_2$.[7] Samples not containing A 23187 or $CaCl_2$ were usually run in parallel.

The in vitro formation of Heinz bodies was obtained upon incubating normal RBCs (containing G6PD B) at a final 50% hematocrit for up to three hours at 37°C in isoosmotic TES containing 20 mmol/L acetylphenylhydrazine (APH). Control samples, not containing APH, were incubated in parallel. Heinz bodies were observed by phase contrast microscopy[17] and express from − to + + + +. GSH was estimated according to Beutler.[18]

RESULTS

RBC proteolytic systems and autoxidation of divicine. Procalpain undergoes significant inactivation in intact RBCs exposed to autoxidizing divicine (Table 1). Loss of procalpain activity is consistently greater in the G6PD-deficient RBCs than in the normal RBCs because of the distinctive lability of GSH in the genetically affected cells.[6] On the contrary, the acid endopeptidase activity of both normal and G6PD-deficient RBCs incubated in the presence of autoxidizing divicine is unaffected.

Proteolytic events in Ca^{2+}-loaded RBCs. Loading of intact RBCs with 100 μmol/L Ca^{2+} by treatment with the A 23187 ionophore is followed by remarkable alterations in both proteolytic systems, ie, the procalpain-calpain system and the three acid endopeptidases normally associated with the membrane.[19] Specifically, procalpain undergoes extensive inactivation (from 20.3 ± 3.6 to 8.7 ± 2.3 units/mL in the normal RBCs and from 22.1 ± 3.2 to 8.1 ± 2.4 units/mL in the G6PD-deficient RBCs). This inactivation is the result of transient conversion of procalpain to calpain that is then degraded to inactive fragments intracellularly.[7] The total acid endopeptidase activity is not substantially decreased, but the enhanced Ca^{2+} levels in RBCs determine an almost complete release of this activity from the membranes (11.9 ± 2.9 units/mL in the untreated cells) into the cytosol (8.2 ± 1.3 units/mL, following incubation of the Ca^{2+}-loaded cells).[7] No appreciable difference is observed between normal RBCs and G6PD-deficient RBCs concerning the extent of solubilization of acid endopeptidase activity (data not shown).

Proteolytic events in RBCs from favic patients. RBCs withdrawn from favic patients are a mixed population of damaged cells, undamaged reticulocytes being released into circulation, and probably a small proportion of mature RBCs lacking significant alterations.[20] Therefore, preliminary removal of reticulocytes by density gradient centrifugation is required for adequate and homogeneous analyses of changes in the RBCs of favic subjects.[2]

Table 2 shows the levels and the subcellular distribution of procalpain (100% cytosolic) and acid endopeptidase activity in the dense RBC fraction from favic patients. Relevant controls (equally cleared of reticulocytes by density gradient centrifugation) were normal RBCs from healthy subjects, RBCs from a posthemorrhagic subject with high reticulocytosis, and RBCs from asymptomatic G6PD-deficient subjects. RBC procalpain activity (4.5 ± 1.5 units/mL) in favic patients is consistently lower than in controls; the acid endopeptidase activity has a reversed subcellular distribution, being much higher in the cytosol (6.0 ± 1.7 units/mL)

Table 1. Intracellular Levels of Procalpain and Acid Endopeptidase Activities in Normal and G6PD-deficient Red Cells Exposed to Autoxidizing Divicine

RBC	Enzyme Activity	Divicine (mmol/L) —	0.5	1.0
Normal	Procalpain	20.3 ± 3.6	15.3 ± 3.2	10.1 ± 2.1
Gd$^-$	Procalpain	22.1 ± 3.2	6.1 ± 4.8	0
Normal	Acid endopeptidase			
	Membrane-bound	11.9 ± 2.9	11.1 ± 2.6	10.7 ± 2.5
	Cytosolic	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.2
Gd$^-$	Acid endopeptidase			
	Membrane-bound	12.2 ± 2.5	12.8 ± 2.9	11.0 ± 2.7
	Cytosolic	0.5 ± 0.1	0.5 ± 0.1	0.8 ± 0.2

Mean ± SD of values from four normal and three asymptomatic G6PD-deficient subjects. All values of procalpain and acid endopeptidase activities are expressed as units/mL of packed RBCs.

Table 2. Levels of Procalpain and Acid Endopeptidase Activities in the Dense RBC Fraction From Favic Patients

Subjects	Retic (%)	RBC Calcium (μmol/L)	Acid endopeptidase (units/mL RBC) Membrane-bound	Acid endopeptidase (units/mL RBC) Cytosolic	Procalpain (units/mL RBC)
Normal (G6PD B)	0.4 ± 0.1	15 ± 4	6.6 ± 1.2	0.6 ± 0.2	15.6 ± 2.5
High reticulocytosis					
G6PD B	7.1	18	7.0	0.7	16.5
Healthy Gd$^-$	0.1 ± 0.05	16 ± 3	7.2 ± 3.6	0.9 ± 0.3	14.8 ± 2.4
Favic during hemolysis					
GS	3.2	230	1.2	6.7	6.2
FS	7.3	215	0.9	7.9	3.4
AP	12.5	390	1.5	5.5	3.1
AC	10.1	120	2.0	3.9	5.3

Reticulocyte percentages refer to the dense RBC fraction. Mean ± SD of values from four normal subjects and four G6PD-deficient subjects; the other values refer to individual patients, ie, the high reticulocytosis G6PD B subject and the four favic patients.

than in the membrane-associated form (1.4 ± 0.05 units/mL). The light RBC fraction from favic patients, containing undamaged reticulocytes and a small portion of oxidant-stressed cells, shows nearly normal procalpain activity, while the acid endopeptidase activity is almost exclusively confined within the membrane (data not shown).

Figure 1 shows the SDS-PAGE patterns of membranes from the same dense RBC fraction (lanes C and D) recovered by density gradient centrifugation of RBCs in a representative favic patient. Extensive formation of dithiothreitol (DTT)-reducible membrane protein aggregates indicates that these cells were subject to severe oxidant stress in vivo, while disproving any major involvement of Ca^{2+}-stimulated RBC transglutaminase activity.[21] Lane A shows the membrane protein profile of the light RBC fraction containing undamaged reticulocytes and obtained from the same patient. Lane B represents the same membrane material run in lane A, following digestion with purified calpain that converts band 2.1 (ankyrin) to band 2.3 and degrades band 4.1 specifically. Comparative inspection of lane D reveals the virtual absence of bands 2.1, 2.3, and 4.1, indicating transient expression of calpain activity in the damaged RBCs as an intermediary step in the conversion of procalpain to inactive calpain fragments (Table 2). Analysis of RBC polypeptide aggregates occurring in favism is feasible by comparing lane C (without DTT) with lane D (with DTT) and by considering the contribution of cytosolic proteins (lane E). The results of such analysis indicate that spectrin, carbonic anhydrase, and hemoglobin (or hemoglobin derivatives) are major components of these disulfide-bridged membrane protein aggregates. The same conclusion is obtained by re-running the high molecular weight material observed on top of lane C (data not shown) on a separate gel under reducing conditions.

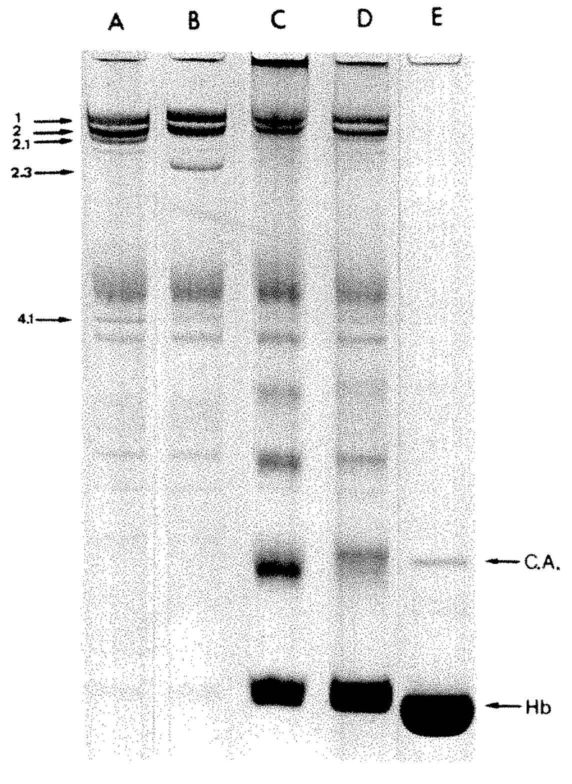

Fig 1. SDS-PAGE of ghosts (lanes A-D) and of the cytosolic fraction (lane E) from RBCs from a favic patient (FS) during hemolytic crisis. (A) Ghosts isolated from the light RBC fraction (containing 34% reticulocytes), following reduction with 40 mmol/L DTT; (B) the same ghosts following incubation for 20 minutes at 25°C with 2 μg of purified procalpain and 100 μmol/L CaCl$_2$; (C) ghosts isolated from the dense RBC fraction; (D) the same ghosts following reduction with DTT; (E) the cytosolic fraction obtained from the dense RBC fraction.

Role of RBC procalpain against oxidant damage. When purified procalpain is allowed to react in the presence of mmol/L Ca^{2+} upon membranes obtained from acetylphenylhydrazine (APH)-treated, ie, from Heinz body-containing normal RBCs, extensive degradation takes place. The proteolytic activity is approximately one half of that observed with the optimal substrate of calpain, ie, acid-denatured globin (Table 3); proteolysis is negligible on white ghosts taken as a control. Figure 2 shows that calpain converts band 2.1 to band 2.3 and degrades band 4.1 selectively in the native membranes[22] (lanes A through C–also Fig 1). On the contrary, band 2.1. to band 2.3 conversion is obscured in the membranes from APH-treated RBCs in which band 3 and hemoglobin chains become preferred targets of proteolytic activity (lanes D through F). Therefore, under conditions of severe oxidative stress, calpain triggers the disruption of both

Table 3. Protein Degradation by Purified Calpain Acting on "In Vitro" Pre-formed Heinz Bodies

Substrate of Calpain	Proteolysis Fluorescence Increase, Arbitrary Units
Membrane, native RBC	12
Membrane, APH-treated RBC (Heinz bodies)	220
Acid-denatured globin	502

Samples (0.25 mL) containing 250 μg of membrane protein (native membrane) or of acid-denatured globin were incubated for 20 minutes at 25 °C in 0.05 mol/L Na borate, pH 7.5, with purified RBC procalpain (2μg) and 1 mmol/L $CaCl_2$. In APH-treated RBCs, the total membrane protein content was 520 μg, 270 μg of which corresponded to denatured hemoglobin.[12]

proteins responsible for the onset of aggregation of Heinz bodies on the RBC membrane.[23]

A protective role of the procalpain-calpain system against oxidant damage seems to be confirmed by results obtained by incubating intact RBCs with 20 mmol/L APH. This represents the optimal concentration eliciting generation of Heinz bodies.[24] In these conditions, progressive Heinz body formation is paralleled by time-dependent decay of RBC procalpain activity and by loss of glutathione (GSH) (Fig 3). Lower APH concentrations result in little formation of Heinz bodies while the extent of procalpain inactivation becomes less remarkable and not sharply different from that occurring in the control RBC.

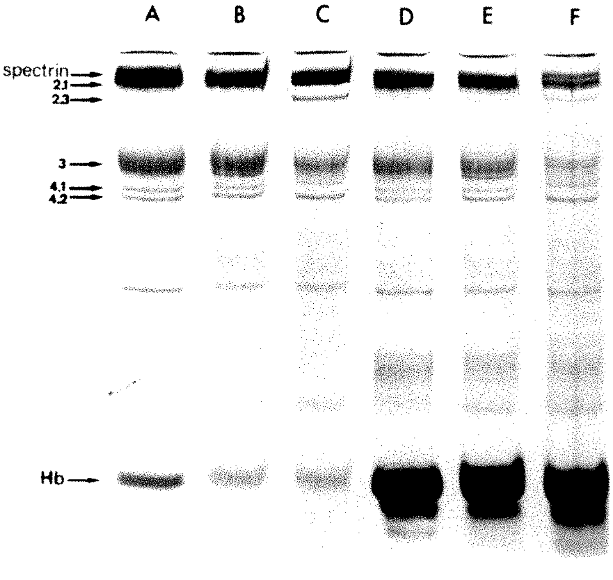

Fig 2. SDS-PAGE of ghosts isolated from normal RBCs following incubation with APH. All samples were reduced with 40 mmol/L DTT before electrophoresis. (A) ghosts isolated from native RBCs incubated for 3 hours at 37°C in isoosmotic TES, pH 7.4; (B) the same ghosts following incubation for 20 minutes at 25°C with 3.5 μg of purified procalpain in the absence of Ca^{2+}; (C) the same ghosts following incubation with purified procalpain in the presence of 100 μmol/L $CaCl_2$; (D) ghosts isolated from RBCs exposed to 20 mmol/L APH in isoosmotic TES for 3 hours at 37°C; (E) ghosts isolated from APH-treated RBCs following incubation for 20 minutes at 25°C with 3.5 μg of purified procalpain in the absence of Ca^{2+}; (F) ghosts isolated from APH-treated RBCs following incubation with purified procalpain in the presence of 100 μmol/L $CaCl_2$.

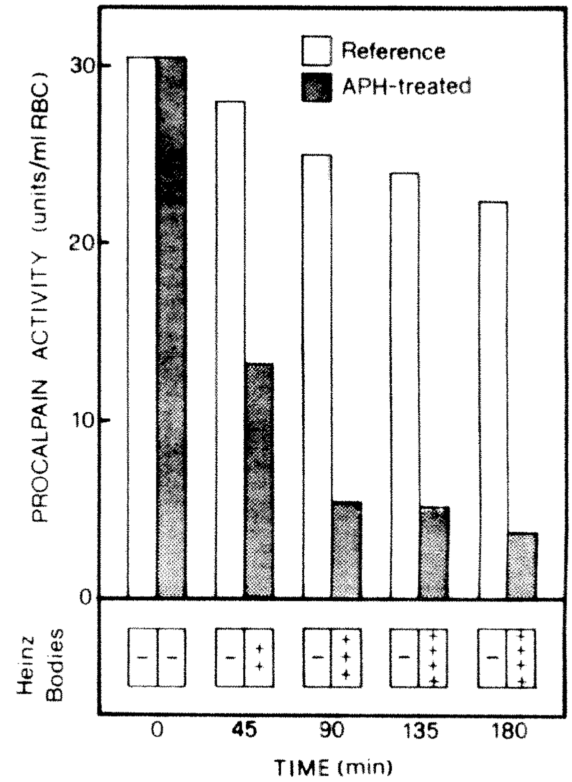

Fig 3. Loss of procalpain activity and Heinz body formation in normal (G6PD B) RBCs exposed to 20 mmol/L APH without glucose. The levels of intracellular GSH in the APH-treated RBC dropped from 8.42 μmoles/g Hb to 0.71 μmoles/g Hb within 90 minutes and to 0.42 μmoles/g Hb at 180 minutes incubation. In the control RBCs, ie, untreated, GSH was completely stable at 90 minutes and dropped to 0.85 μmoles/g Hb after 180 minutes incubation. Values refer to a representative experiment.

DISCUSSION

The procalpain-calpain system of mature RBCs has unique properties that make it highly susceptible to a number of inactivating agents in the intracellular environment. Thus, besides inactivation by autoxidizing divicine,[6] which apparently involves oxidation of cysteine residue(s) at the active site of the enzyme,[25] mobilization of Ca^{2+} is known to cause transient conversion of procalpain to calpain and eventually an autoproteolytic mechanism of inactivation. Alternative mechanisms for the formation of calpain have been described, such as binding of procalpain to the RBC membrane and the availability of abnormal protein substrates that reduce the concentration of Ca^{2+} required for the conversion of procalpain to calpain.[25] In all cases, whatever the mechanism of formation, active calpain undergoes autoproteolysis to yield inactive fragments.

RBCs in G6PD-deficient patients during hemolysis from favism show abnormally low procalpain activity, predicted because of oxidant stress exerted by divicine autoxidation[6] and the late impairment of Ca^{2+} homeostasis within RBCs.[1,2,7] Another major alteration found in favism, which can be ascribed to enhanced intracellular Ca^{2+},[7] affects the acid endopeptidases in which subcellular distribution is almost completely reversed. If, as suggested,[25] a major role of the acid endopeptidases is the eventual membrane-directed breakdown of partially nicked cytosolic proteins, their release into the cytosol may be equivalent to functional switching off. Data reported in Table 2 rule out any obvious correlation in the individual favic patients between the intracellular Ca^{2+} levels actually measured in RBCs and the extent of alterations affecting procalpain and the acid endopeptidases. This lack of correlation is easily explained in the case of procalpain, which is a target of two inactivating agents, divicine autoxidation and perturbed Ca^{2+} homeostasis. No immediate explanation, however, is apparent for the loose quantitative relationship between solubilization of acid endopeptidases (the mechanism of which is still undefined)[7] and actual Ca^{2+} concentrations in the affected RBC. Local changes in the concentrations of Ca^{2+},[26] such as those involving its compartmentalization in intracellular inside-out vesicles within sickle cell anemia RBCs,[27] could be responsible for this poor correlation.

Selective breakdown of band 3, and of glycophorin as well, has been observed following loading of RBCs with high Ca^{2+} concentrations, most probably as a result of transglutaminase-activated polymerization process.[28] In favism, the intraerythrocytic Ca^{2+} levels are generally below the threshold of 0.4 to 0.5 mmol/L known to trigger the endogenous transglutaminase activity,[21] but certainly high enough, ie, above 100 μmol/L,[7] to promote quantitative release of acid endopeptidase from the membrane into the cytosol (Table 2). Moreover, the intracellular Ca^{2+} levels found in favic patients are widely sufficient to induce conversion of procalpain to calpain and the eventual autoproteolytic inactivation of the active enzyme species. This activation-inactivation mechanism has been shown to occur in vitro at consistently lower Ca^{2+} concentrations than those found in favism,[1,2] provided that digestible protein substrates are available.[25] Therefore, loss of procalpain activity may be viewed not merely as reflecting the late impairment of Ca^{2+} homeostasis, but also as an early step in RBC damage, when nascent protein aggregates are formed because of autoxidation of divicine and intracellular Ca^{2+} levels are still low. Experiments with membranes from APH-treated RBCs (Fig 2) indicate that the nucleation process leading to Heinz body formation is to some extent counteracted by calpain or by some proteolytic process, eg, catalyzed by acid endopeptidases, initiated by calpain activity. The oxidant stress inherent in divicine autoxidation appears to trigger nucleation of nascent protein aggregates on the membrane; at the same time and by various mechanisms the RBCs are deprived on the proteolytic system that prevents the irreversible transition of these aggregates to Heinz bodies. This conclusion emerges from the inverse relationship between procalpain inactivation and Heinz body formation in the APH-treated RBCs (Fig 3).

The present findings support the view that procalpain is a defense system, which is normally latent in RBCs, but which can be activated by a variety of mechanisms that protect against an oxidant challenge. The role of calpain in producing limited degradation of excess globin chains to be eventually broken down by acid endopeptidase activities in RBC membranes has been previously demonstrated, thus implying its active involvement in thalassemias.[29] Such involvement is also suggested by the recent finding of high Ca^{2+} levels in β-thalassemic RBCs.[30] Although these high levels are now recognized to affect reticulocytes and circulating nucleated cells rather than dense RBCs from β-thalassemic patients,[31] the presence of globin aggregates might reasonably decrease the amount of Ca^{2+} required to activate procalpain.[25] The peculiar susceptibility of procalpain to divicine autoxidation in G6PD-deficient RBCs, coupled with the multiple mechanisms leading to its transient activation and late inactivation, suggests that exhaustion of this proteolytic system is a crucial step in the onset of RBC damage in favism, resulting in unrestrained Heinz body formation.

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Spectrin Nice ($\beta^{220/216}$): A Shortened β-Chain Variant Associated With an Increase of the $\alpha^{I/74}$ Fragment in a Case of Elliptocytosis

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We describe a new spectrin variant with a truncated β-chain. It was discovered in a 17-year-old white boy presenting with intermittent jaundice and spleen enlargement. He also displayed numerous smooth elliptocytes. On sodium dodecyl sulfate–polyacrylamide gel, the truncated β-chain (β'-chain) appeared as an additional band of approximately 216 kilodaltons, migrating between spectrin β-chain and ankyrin. It represented 30% of total β-chain. The β'-chain reacted with an antispectrin β-chain monoclonal antibody. It failed to become phosphorylated when ghosts were incubated in the presence of [γ-^{32}P] adenosine triphosphate. Whole spectrin tetramerization was defective since the amount of spectrin dimer was increased in spectrin crude extract and the association constant of the spectrin dimer self-association was decreased. Spectrin whole tetramer isolated from spectrin crude extracts contained small quantities of β'-chain. Spectrin tryptic peptides showed an increase of the 74,000-dalton fragment at the expense of the 80,000-dalton fragment. So far, the latter abnormality has been used to characterize a number of cases of hereditary elliptocytosis or pyropoikilocytosis with no other apparent change. In the present case, we consider that the abnormality is a consequence of the β-chain alteration. The parents seemed asymptomatic. As a result, we regard this new spectrin variant as deriving from a *de novo* mutation.

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HEREDITARY elliptocytosis (HE) refers to a variety of conditions defined on the basis of a common morphological feature. Yet, these conditions are heterogeneous. The heterogeneity results from the genetic polymorphism of membrane skeletal proteins.[1] So far, most of the molecular alterations responsible for HE seem to be distributed in two distinct areas of the skeleton. Some HE are due to a partial or total lack of protein 4.1[2-4] or to some qualitative alteration of this protein.[5] Other HE are the consequence of changes of the head of spectrin. Several types of α-chain abnormalities have been described on the basis of altered peptide patterns and referred to as Sp$\alpha^{I/74}$,[6,7] Sp$\alpha^{I/46}$,[6,8] and Sp$\alpha^{I/65}$.[9,10] In contrast, modifications of spectrin β-chain appear more sporadic, if not exceptional. Two shortened variants have been described in detail.[11,12]

This work describes a spectrin variant with a short deletion and elliptocytosis. The abnormal β-chain lacks a fragment of 4 kilodaltons (kDa) approximately. The abnormal spectrin displayed an increase of the $\alpha^{I/74}$ peptide following tryptic digestion.

MATERIALS AND METHODS

Case report. The propositus (F.B.) was 17 years old when he was first examined by us in 1984. He complained of asthenia and abdominal pains, the latter developing after meals or sports. For several years, he had been suffering from intermittent jaundice coinciding with the pains. Soon after birth he had presented with an acute hemolytic episode requiring a blood transfusion. Upon physical examination, he appeared normally developed. The spleen was enlarged: 14 × 10 × 6 cm, as determined by echography. X-ray film findings from the chest and the skull were normal. The first routine laboratory analyses showed the absence of anemia (Hb, 152 g/L), an increased reticulocyte count (260×10^9/L, 5.5%), and increased total bilirubin (70 μmol/L). Blood smears revealed almost 100% elliptocytosis, as will be detailed. A few days later, F.B. was admitted to the hospital for acute abdominal pains and diarrhea. Icterus was aggravated. Laboratory analyses showed a reduced hemoglobin level (135 g/L), a further increased reticulocyte count (390×10^9/L, 9%) and increased total bilirubin content (149 μmol/L). The haptoglobin concentration was low (0.10 g/L). There was significant urobilinuria but no hemoglobinuria. The Coombs' test and the test of Ham and Dacie were negative. Osmotic resistance was normal (initial hemolysis, 0.51% NaCl [normal, 0.48%]; total hemolysis, 0.39% NaCl [normal, 0.36%]). However, the acidified glycerol test was abnormal (OD_{50} attained after 130 seconds *v* 1,800 seconds in controls). Red cell half-life was 9.5 days, as determined with ^{51}Cr. There was significant splenic sequestration. The bone marrow displayed numerous erythroblasts. Hemoglobin, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and hexokinase levels were normal. Pentoxifylline (Trental) alleviated the abdominal pains and has prevented the recurrence of acute manifestations since this episode. The parents and one brother were clinically and hematologically normal. On the basis of HLA and RBC group typing using the Bay method, paternity could not be excluded, with a probability of between .977 and .997.[13] Many investigations carried out in the propositus and the parents were also performed in the propositus' brother: they yielded normal results in the latter and will not be detailed here.

Study of erythrocyte morphology, deformability, and thermal sensitivity. Blood samples were collected on a citric acid–citrate medium kept at ice-water temperature and treated within 24 hours. Red cell morphology and thermal sensitivity were determined as described previously.[8] Erythrocyte deformability was measured by using ektacytometry as a function of osmolality.[14]

Preparation of erythrocyte ghosts and crude spectrin extract. Red cell ghosts were prepared essentially according to

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Dodge et al[15] except that phenylmethylsulfonylfluoride (PMSF) (0.3 mmol/L) was added to the lysis buffer. In a control experiment, the membranes were prepared with diisopropyl fluorophosphate (DFP) according to Marchesi et al.[16] Crude spectrin extract was released from ghosts by incubation in a low–ionic strength buffer[17] containing 0.3 mmol/L phosphate (pH 8.00), 0.1 mmol/L PMSF, and 0.1 mmol/L EDTA, 0.1 mmol/L β-mercaptoethanol (β-ME). Incubation was performed at 4°C overnight or 30 minutes at 37°C.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis. SDS-PAGE was performed according to the procedure of Laemmli[18] or the procedure of Fairbanks et al.[19] The procedure and the acrylamide concentration in each experiment will be indicated in a different section or in the figure legends. Gels were stained with Coomassie blue and scanned at 570 nm.

Immunologic characterization of abnormal spectrin chain. Monoclonal antibodies directed against human red cell membrane spectrin were prepared by immunizing BALB/c mice with chromatographically purified spectrin.[20] Fusion of spleen cells with Sp2/OAg 14 myeloma cells, hybridoma selection, and cloning were done by using the single-step technique described by Davis et al.[21] Screening of hybridoma supernatants was done by using an enzyme-linked immunosorbent assay with purified spectrin as the solid-phase antigen.[22] The supernatants of nine different clones were found to be positive. Three of the nine clones were expanded in culture, and the antibodies were further characterized with an immunoblotting technique (described later). One clone (AD5) produced an antibody directed against spectrin α-chain, and two clones (CA5 and BD6) produced antibodies directed against spectrin β-chain. Immunoblotting was carried out essentially as previously described.[5,23]

Phosphorylation of membrane proteins. Phosphorylation was carried out according to a technique derived from that of Roses and Appel[24] with some modifications.[25]

Whole spectrin dimer–dimer association. Crude spectrin extract (4°C) was analyzed to deduce the spectrin dimer (SpD)/SpD + spectrin tetramer (SpT) ratio as described by Liu et al[26] with some modifications.[27] Crude spectrin extract (37°C) was submitted to tetramerization according to a technique derived from that of Ungewickell and Gratzer[28] and adapted by Alloisio et al.[27] The latter mixture, which will be referred to as crude spectrin extract (37°C, tetramerized), was analyzed to deduce the equilibrium constant Ka.[26] A kinetic analysis of the dimer-tetramer conversion was also carried out at the following times: 0, 1, 2, 3, and 4 hours.

Distribution of the β'-chain in SpT and SpD. To determine the ability of $\alpha\beta'$-dimer to undergo polymerization, SpT and SpD from 4°C and 37°C extracts (37°C, tetramerized) were first separated in nondenaturing gels, as described. Bands corresponding to the various spectrin species were visualized by staining the gels according to Cleveland et al.[29] Then they were cut off and rerun using SDS-PAGE.

Spectrin peptide maps. Crude spectrin extract (37°C) was dialyzed for one hour against 10 mmol/L phosphate buffer (pH 8.00) and 40 mmol/L NaCl at 4°C and adjusted to 1 mg/mL. The preparation was then left at 4°C for another two hours. Samples were then digested for 20 hours at 4°C in the presence of trypsin at a concentration of 1% (wt/wt). The digestion was stopped with 2 mmol/L PMSF. In an experiment dealing with spectrin Le Puy ($\beta^{220}/^{214}$), a distinct shortened β-variant described previously,[11] the preincubation and digestion were carried out under slightly different conditions.[8,9] We also carried out a kinetic analysis of the trypsin digestion of the present variant. One- and two-dimensional electrophoresis was performed with the procedure used by Speicher et al.[30]

Hemolysate/ghost cross-incubations. To relate the deletion on the β-chain to a protease activity, we carried out the following cross-incubations: mother's hemolysate (or red cell cytosol)/father's ghosts and father's hemolysate (or red cell cytosol)/mother's ghosts. Hemolysate was obtained by mixing 2 vol of water with 1 vol of washed red cells. The cytosol was obtained by centrifugation (20,000 rpm, 20 minutes). Ghosts were prepared as previously indicated. Cross-incubations were carried out at 37°C for three hours in 5 mmol/L phosphate buffer (pH 7.4), 150 mmol/L KCl (hemoglobin/ghost ratio, 200/1 [wt/wt]). After three washings with 5 mmol/L phosphate buffer (pH 8.00), the ghost proteins were analyzed with SDS-PAGE according to Fairbanks et al[19] with a 3.5% to 17% exponential acrylamide gradient.

RESULTS

Most of the experiments concerning the propositus have been repeated on at least two blood samples. Except for Table 3, the results of one experiment are presented. Unless otherwise stated, means are given as $\overline{m} \pm 2\,\sigma$.

Morphology, deformability, and thermal sensitivity of the red cells. Examination of erythrocytes on dried blood smears or after fixation with glutaraldehyde gave consistent results. In the propositus, elliptocytosis (95% of the cells) was prominent and associated with a marked anisocytosis (not shown). The elliptocytes were long and smooth and had no tendency to bud or fragment. Red cells from both parents had a normal morphology. Upon osmotic gradient ektacytometry, the curve was abnormal in the propositus. The index was significantly decreased in isotonicity (−5.7 SD from the mean value of normal adults). The maximum index was displaced toward hypotonicity (Fig 1). The curves were normal in the parents. Erythrocyte thermal sensitivity was slightly abnormal in the propositus. No fragmentation occurred at 45°C; however, at 47°C, 5% of the red cells were budding after 40 minutes. This result was consistent with the abnormal decrease in EI observed after heating at 47°C for 20 and 40 minutes (not shown). In the parents, all values were normal, and no fragmentation was observed at 47°C.

Analysis of total membrane proteins. SDS-PAGE of the red cell membrane proteins allowed us to isolate an additional band in the propositus (Fig 2). It stood between spectrin β-chain and ankyrin, with a molecular weight of approximately 216 kDa. It accounted for approximately 30% of the total β-chain and appeared clearly at the expense of normal β-chain (Table 1). No other change was detectable in

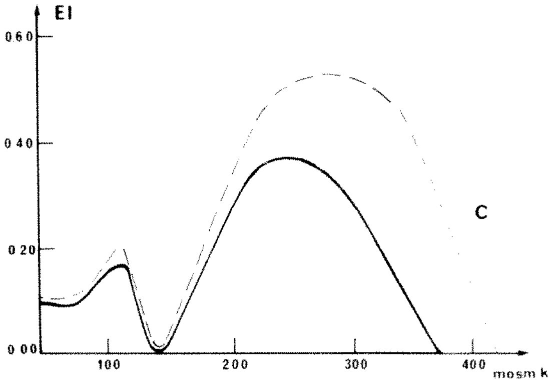

Fig 1. Osmotic gradient ektacytometry. Ektacytometric index (EI) was measured during a progressive increase of the suspending medium osmolality. C, control.

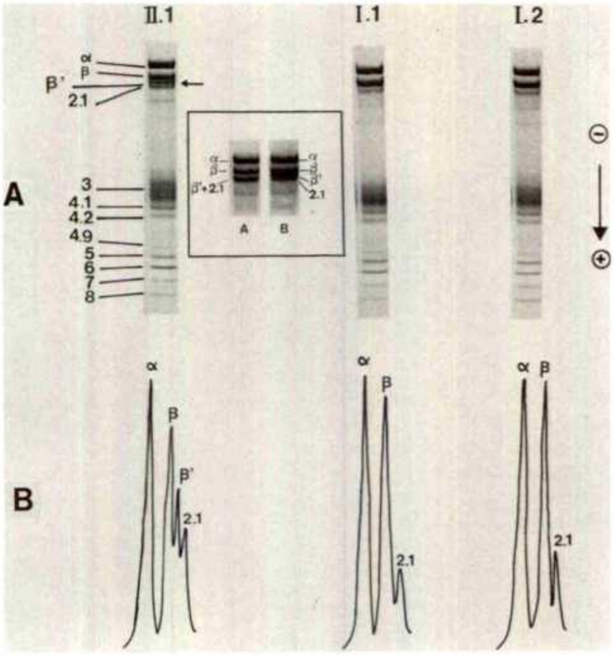

Fig 2. Electrophoretic pattern of total membrane proteins. (A) SDS-PAGE according to Fairbanks et al[19] by using a 3.5% to 17% exponential gradient of acrylamide. Arrow, additional β' band. (B) Densitometric profile of the spectrin region. In II.1, the apparent increase of band 2.1 is the consequence of the overlapping of band β'. Inset, comparative migration of (A) spectrin Le Puy ($\beta^{220/214}$)[11] and (B) the present abnormal spectrin.

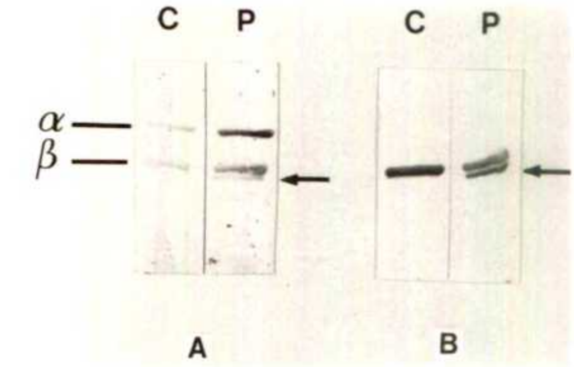

Fig 3. Immunologic studies. SDS-PAGE was carried out according to Laemmli[18] by using a 3.5% to 17% acrylamide gradient. Transfer was performed on nitrocellulose sheets. (A) Ponceau red staining. (B) Immunoblot. C, control; P, patient; ←, β'-chain.

the propositus. The variant appeared without exception and with fixed proportions in the four blood samples examined. In addition, it was present when proteins were prepared with strong protease inhibitor or when whole red cells were directly treated with SDS buffer prior to electrophoresis. In the parents, the membrane protein profile was normal.

Immunoblots. For immunologic identification of the additional band, it was mandatory to separate the band from the β-chain (Fig 3). Immunoblot of the propositus membrane proteins was performed with two monoclonal antispectrin β-chain antibodies. The shortened β-chain was stained with the monoclonal antibody BD6. This showed that the variant chain derives from the β-chain. However, it was not recognized by the monoclonal antibody CA5. This discrepancy in the behavior of the two antibodies could mean that antibody CA5 is directed against the fragment of spectrin β-chain that is deleted in the shortened variant.

Phosphorylation of red cell membrane proteins. Again, it was necessary to separate the β- and β'-chains as much as possible (Fig 4). Under these conditions, no labeling appeared in the place of the β'-chain. To confirm this observation, the region of the gel corresponding to the β'-chain was cut out in the propositus and in a control and submitted alone to autoradiography. The labeling of the β'-piece was not higher than the background (not shown). This experiment showed that the deletion removes (or at least blocks access to) the phosphorylation sites that are near the COOH terminus of the β-chain.[31]

SpD self-association. Whole spectrin tetramerization was defective in the propositus. An estimation of the native spectrin species in crude spectrin extract (4°C) showed an increase in the SpD/(SpD + SpT) ratio (Table 2). Spectrin self-association studied in crude spectrin extract (37°C) was found defective as shown by the decrease of the association constant (Ka). Although in the father the SpD/(SpD + SpT) ratio in crude spectrin extract (4°C) was slightly increased and Ka was found just below normal values, these borderline differences failed to be confirmed by kinetic analysis of the dimer-tetramer conversion (not shown). All values were normal in the mother.

The nondenaturing gels used for these experiments provided additional data concerning the presence of β'-chain in whole dimer, tetramer, and oligomer. In the propositus' crude spectrin extract (37°C) there was a faint satellite band near the tetramer (not shown). This band was best seen before tetramerization, the tetramer band being narrow. After tetramerization, some oligomer appeared: it was also duplicated in the propositus. We assume that the new bands correspond to a subpopulation of tetramer and oligomer that contains the abnormal β'-chain. No additional β' species appeared near the normal dimer, presumably because the

Table 1. Quantitative Study of Spectrin Subunits From Densitometric Profiles of Fairbanks' Gels

	Controls	Propositus	Father	Mother
$\alpha/3 \times 100$	49.3 ± 4.8 (n = 8)	46.2	51.4	50.8
$\beta/3 \times 100$	43.8 ± 4.4 (n = 8)	30.2	44.6	45.5
$\beta'/3 \times 100$	—	14.5	—	—
$\beta + \beta'/3 \times 100$	—	44.7	—	—
$\beta'/\beta + \beta' \times 100$	—	32.4	—	—

Spectrin bands were determined with respect to band 3.

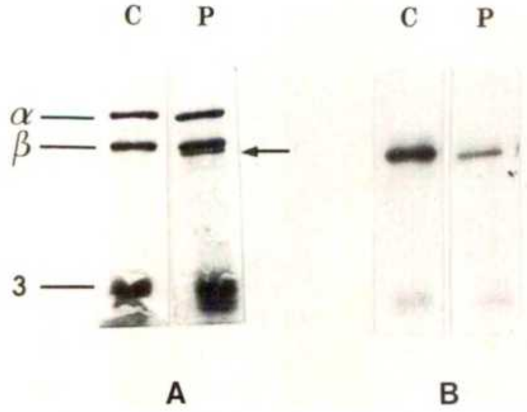

Fig 4. Phosphorylation of membrane proteins without cyclic adenosine monophosphate. SDS-PAGE was carried out according to Laemmli[18] by using a 3.5% to 17% acrylamide gradient. (A) Stained gel; the β'-chain has been clearly separated (←). (B) Autoradiogram; the β'-chain is not labeled.

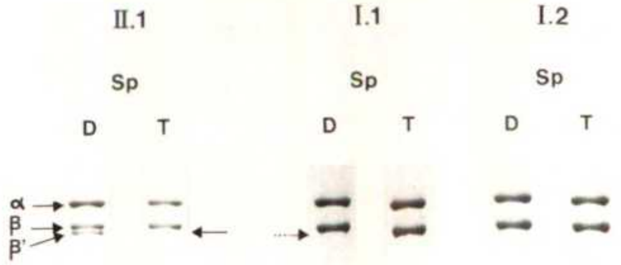

Fig 5. Distribution of β- and β'-chains in SpD and SpT. Crude spectrin extract (37°C, retetramerized) was run on nondenaturing gels. SpD (dimers) and SpT (tetramers) were then cut out and rerun with SDS-PAGE according to Laemmli[18] with a 3.5% to 17% acrylamide gradient. In II.1, most of the β'-chain appeared in SpD; however, there was some β'-chain in SpT also (←). There was a faint band below the β-chain in SpD from I.1 ( ); its nature remained unknown.

difference in charge and/or molecular weight was present one time only and therefore could not be resolved. No satellite band was visible in the parents. This experiment showed that the β'-dimer is not completely unable to self-associate.

Presence of β'-chain in SpD and SpT. The distribution of β- and β'-chains was studied in SpD and SpT. After an initial electrophoresis on nondenaturing gels, the bands corresponding to SpD and SpT were cut out and rerun using SDS-PAGE (Fig 5). Although most of the β'-chain was present in SpD, there was invariably a small amount of the β'-chain in SpT, whatever the type of extract: 13%, 19%, and 14% in crude spectrin extract at 4°C, 37°C, and 37°C (retetramerized), respectively. This finding is to be related to the SpT satellite band seen on nondenaturing gels. The β' proportion varied depending on the type of extract; however, the approximate amount of β'-chain appearing in the SpD and SpT remained quite constant.

Spectrin peptide maps. Samples were matched with controls run simultaneously. On one-dimensional gels, the propositus repeatedly displayed a reduction of the αI 80,000-dalton fragment at the benefit of a 74,000-dalton fragment (Fig 6, Table 3). Densitometric analysis showed that the 74,000-dalton fragment derived from the 80,000-dalton fragment since the sum of both peaks remained constant. Therefore, it is unlikely that the increase would result from contamination with the $\beta^{IV/74}$ peptide. In addition, two-dimensional electrophoresis showed that the $\alpha^{I/74}$ spot was more pronounced (at the expense of the $\alpha^{I/80}$ spot) and that the $\beta^{IV/74}$ peptide appeared normal (not shown).

Table 2. Whole Spectrin Dimer-Dimer Association

	$\frac{SpD}{SpD + SpT} \times 100$	Ka × 10^5 L mol^{-1}
Propositus	39.2 ± 3.58*	1.6 ± 0.28†
Father	11.6 ± 1.74*	3.4 ± 0.32†
Mother	7.7 ± 1.56*	4.2 ± 0.24†
Controls	6.6 ± 3.57 (n = 9)	4.6 ± 1.10 (n = 23)

The spectrin dimer proportion, SpD/(SpD + SpT), was determined in the spectrin crude extract (4°C). The association constant (Ka) was determined from the spectrin crude extract (37°C).

*Five determinations on the same sample.

†Four determinations on the same sample.

Concerning the increase of the $\alpha^{I/74}$ peptide, additional experiments were carried out. (1) A kinetic analysis of trypsin digestion was performed (Fig 7). In the controls as in the propositus, the 74,000-dalton peptide appeared clearly after 20 minutes of digestion. The 74,000/(80,000 + 74,000) ratio increased gradually. Whatever the time, it was constantly higher in the propositus than in the controls. (2) We investigated the status of the $\alpha^{I/74}$ peptide in spectrin Le Puy ($\beta^{220/214}$), a previously described spectrin variant carry-

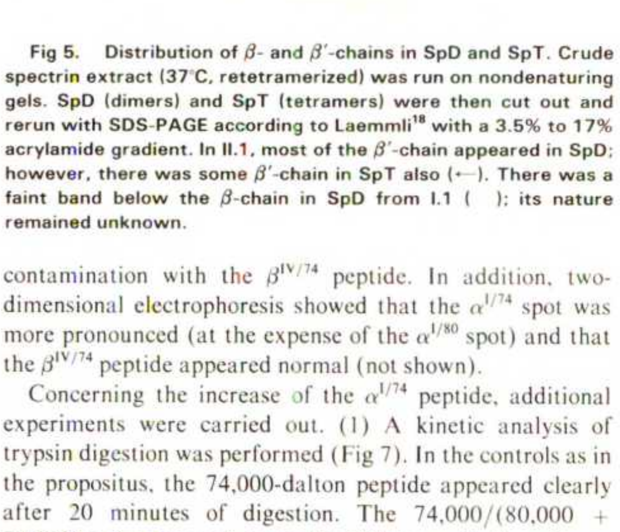

Fig 6. One-dimensional maps of spectrin tryptic peptides. (A) SDS-PAGE was carried out according to Laemmli[18] with a 10% to 15% acrylamide gradient. (B) Densitometric profile to the region of 80,000- to 74,000-dalton peptides. The 80,000-dalton fragment corresponds to the αI domain. The 17,000- and 11,000-dalton fragments corresponds to the βI domain.[30] In II.1, the increase of the 74,000-dalton fragment occured at the expense of the 80,000-dalton fragment (Table 3), and the 17,000- and 11,000-dalton fragments were normal.

Table 3. Quantitative Determination of the 80,000- and 74,000-Dalton Fragment From Densitometric Profiles on One-Dimensional Maps

	$\frac{74}{74 + 80} \times 100$		$\frac{80}{(74 + 80)} \times 100$	
Determination	Propositus	Controls	Propositus	Controls
I	44.5	28.7 ± 0.85 (n = 3)	10.1	11.1 ± 1.0 (n = 3)
II	43.4	30.7 ± 1.13 (n = 3)	14.5	14.2 ± 2.01 (n = 3)
III	45.2	28.1 ± 3.4 (n = 3)	11.5	12.2 ± 0.60 (n = 3)

In the propositus, three different digestions were carried out (two in one sample, one in another sample).

ing a larger deletion on the β-chain.[11] In this experiment, the preincubation and the trypsin digestion were performed under slightly different conditions,[8,9] and the amount of the $\alpha^{I/74}$ peptide in the controls was lower (8% to 10%). Nonetheless, the percentage of the 74,000-dalton fragment in spectrin Le Puy appeared, again, increased (33%). It may be noted that the increase was not as pronounced as in a case of "conventional" Sp$\alpha^{I/74}$ HE encountered in a black patient and run in parallel to spectrin Le Puy; we found 41%.

There was no other alteration of spectrin peptide maps, especially using two-dimensional analysis (not shown). In particular, the βI domain appeared normal, in the form of spots at 17,000 and 11,000 daltons (not shown). We are aware that the portion of the βI domain carrying the phosphorylation sites and therefore the deletion cannot be visualized by this technique.[30] The one- and two-dimensional patterns were normal in the parents.

Cross-incubations. Incubation of mother's hemolysate (or red cell cytosol) with father's ghosts failed to elicit the β'-chain (not shown). The reverse experiment (father's hemolysate (or cytosol) *v* mother's ghosts) was negative. There was no nonspecific proteolysis during the incubations.

DISCUSSION

This study deals with a third truncated variant of spectrin β-chain, following previous reports by Dhermy et al[11] and Ohanian et al.[12] The present variant is different from spectrin Le Puy ($\beta^{220/214}$).[11] The latter has a lower molecular weight (214 kDa), as is shown in Fig 2 (inset), generates budding elliptocytes (even prior to splenectomy), and was dominantly transmitted. It seems different from the variant described by Ohanian et al.[12] The latter has a lower molecular weight (214 kDa). It does not tetramerize and is associated with no increase of the 74,000-dalton fragment. On the contrary, the three variants display a tetramerization defect. In the present case, the dimer displays some ability to tetramerize, at least in vivo. The three mentioned variants also fail to undergo enzymatic phosphorylation, which indicates that the deletion involves the COOH terminal region.[31] If lack of phosphorylation reflects the absence of the phosphorylation sites (and not merely a modified access), the present deletion concentrates these sites onto a smaller segment of the β-chain than observed with previous variants.

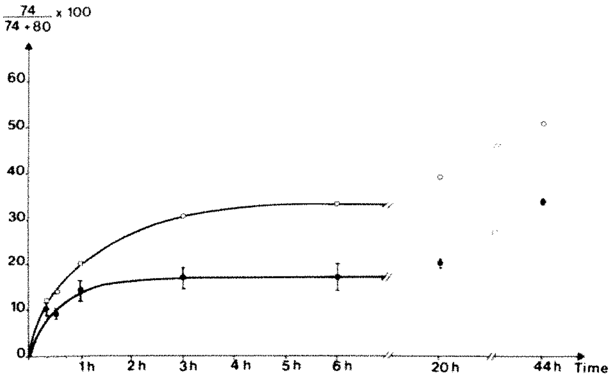

Fig 7. Kinetic analysis of trypsin digestion of spectrin. Spectrin was digested for increasing periods of time to follow the development of the 74,000-dalton fragment. ○, propositus; ●, controls (n = 3).

In the present family, the parents are apparently free of the spectrin abnormality. Although the father displayed borderline variations of the tetramerization parameters, this was not confirmed by tetramerization kinetic analysis. The variant would appear, therefore, as the result of a de novo mutation. The variant described by Ohanian et al[12] also appears as the result of a de novo mutation (although correct attribution of parentage was not definitely established).

The possibility exists that the molecular lesion is an amino acid substitution that creates a new proteolytic site. As a result, the question was raised as to whether the deletion exists in vivo. Using ghosts prepared in the presence of PMSF and DFP,[16] we observed that the variant is present in the same proportions. In addition, the variant is also present in whole red cells directly treated with SDS buffer prior to electrophoresis. These data indicate that the deletion is not an experimental artifact and that ectopic proteolysis (considering the hypothesis of an additional proteolytic site) must occur in situ. However, a reticulocyte control was not performed on the patient to see whether the proportion of shortened β-chain was lower, a fact that would have supported the view of a proteolytic event still operating in the young red cell. The negative results obtained with cross-incubations did not support the idea that the mutation was silent in one parent and was revealed in the propositus by some specific protease activity transmitted by the other parent.

The most critical point of this work is probably the increase of the 74,000-dalton fragment associated with the β-chain deletion. Although the relative amount of the 80,000- and 74,000-dalton fragments may slightly vary from one experiment to another, depending on a conformational equilibrium (Dhermy et al, unpublished data), an increase of the 74,000-dalton fragment was found, without exception, in the propositus *v* the corresponding controls and at each time during the digestion kinetic analysis. In the common Sp$\alpha^{I/74}$

abnormality, the increase of the 74,000-dalton fragment is transmitted from one generation to another, even though this abnormality is not always associated with HE or hereditary pyropoikilocytosis (HPP)[1] (also unpublished data). In the present case, the parents display a normal 74,000-dalton fragment. We therefore assume that the increase of this fragment in the propositus is dependent on the β-chain deletion. This assumption is strongly substantiated by the observation of similarly increased $\alpha^{I/74}$ material in spectrin Le Puy ($\beta^{220/214}$), another spectrin variant with a deletion on the β-chain.[11] One extreme possibility would be that other reported cases of increased 74,000-dalton fragments would be the consequence of (yet unidentified) β-chain alterations. It is known that isolated α-chain from normal subjects yields high amounts of the 74,000-dalton peptide and that the isolated abnormal $\alpha^{I/74}$ chain from previously reported HE and HPP cases behaves as the control.[6,32] The $\alpha\beta$-dimer is abnormal, but the isolated α-chain seems normal. A normal β-chain may be necessary for the α-chain to assume a correct conformation.

This new variant of spectrin β-chain is a truncated variant. The deletion (−4 kDa) involves the region that carries the phosphorylation sites. It yields elliptocytosis and alters spectrin dimer self-association. The missing fragment would result in a conformational change of the α-chain. While awaiting the identification of the fine structural alteration, we suggest that the present variant be termed spectrin Nice ($\beta^{220/216}$).

ACKNOWLEDGMENT

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Transfusion-Associated AIDS: Donor–Recipient Human Immunodeficiency Virus Exhibits Genetic Heterogeneity

By A. Srinivasan, D. York, P. Ranganathan, R. Ferguson, D. Butler, Jr, P. Feorino, V. Kalyanaraman, H. Jaffe, J. Curran, and R. Anand

The genetic diversity of the human immunodeficiency virus (HIV) isolated from transfusion-associated AIDS patients has been examined. Restriction enzyme mapping studies of integrated proviral DNA of donor and recipient origin demonstrated genomic variation between isolates. Analysis of the molecularly cloned viral genomes of one donor-recipient pair showed that virus from the recipient had restriction enzyme site differences from the donor, noticeably clustered in the *env* and *orf*-2 regions, and also had a greater number of restriction sites in common with the donor as well. These results suggest that HIV may undergo genomic variation in vivo. Comparison of donor-recipient viruses may further the understanding of the molecular basis for AIDS pathogenesis.

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ACQUIRED immunodeficiency syndrome (AIDS) and related immunologic disorders have been etiologically linked to human immunodeficiency virus (HIV), which is also known as human T-lymphotropic virus type III (HTLV-III),[1] lymphadenopathy-associated virus (LAV),[2] or AIDS-associated retrovirus (ARV).[3] HIV is cytopathic and has tropism for the T4 helper lymphocyte subset.[1-4] Molecular cloning of different HIV isolates led to the charaterization of the genome by restriction enzyme mapping,[5-7] and comparison of the primary nucleotide sequences further revealed a variation of 1.8% between HTLV-III and LAV and up to 9.3% between LAV and ARV-2.[8-11] Restriction enzyme polymorphism studies carried out with a number of HIV isolates from persons not epidemiologically linked to each other showed an altered restriction pattern from one isolate to another.[7,12-14]

The genetic variation observed with HIV isolates from the natural population could result from high mutability of the genome. To examine the extent of genetic changes, we have focused our attention on HIV isolated from transfusion-associated AIDS patients between 18 and 31 months after they had received blood and the blood donors who were the source of their infection. Here we report that virus isolated from blood recipients had restriction enzyme patterns that were different from the patterns of virus from their blood donors.

MATERIALS AND METHODS

Cells and viruses. HIV were isolated from samples obtained from transfusion-associated AIDS patients and their blood donors as described.[15,16] Briefly, peripheral blood collected was subjected to a Ficoll-Hypaque gradient for collection of mononuclear cells. The cells were grown in RPMI 1640 (GIBCO, Grand Island, NY) containing 10% fetal calf serum and 10 μg/mL phytohemagglutinin (PHA, GIBCO) for three days and were further propagated in RPMI medium containing T cell growth factor and 1:5,000-diluted goat antibody to human interferon (Miles Scientific, Naperville, IL). Periodically the culture was tested for virus production by cocultivation with 3-day-old PHA-stimulated normal adult T cells. Cell-free supernatants from the cocultivated cultures were analyzed for retrovirus by reverse transcriptase assay,[1] and virus stock was frozen after two passages in PHA-stimulated peripheral blood lymphocytes (PBLs).

Restriction enzyme analysis. High-molecular weight DNA was extracted from 7-day-old virus-infected adult T cells.[17] DNA (10 μg) was digested with different restriction enzymes and subjected to electrophoresis on a 0.8% agarose gel. The DNA fragments were transferred to nitrocellulose paper and hybridized to a nick-translated HIV full-length genomic probe.[18] The hybridization was performed at 42°C for 18 hours in a buffer containing 50% formamide, 5× SSC, (1 × SSC = 0.15 mol/L NaCl and 0.015 mol/L Na_3-citrate, pH 7), 5× Denhardt's solution (1 mg/mL bovine serum albumin, polyvinylpyrrolidone, and Ficoll), 20 mmol/L $NaPo_4$ (pH 6.5), 100 μg/mL salmon sperm DNA, and 10% sodium dextran sulfate as described.[18] The filters were washed at 65°C with 0.1× SSC, 0.1% sodium dodecyl sulfate, and exposed to Kodak XAR-5 film for one to two days.

Construction and screening of recombinant phage library. Hirt DNA,[19] extracted from adult T cells 72 hours after virus infection, was digested with *Sac*I and fractionated on 10% to 40% sucrose density gradients.[18] Fractions containing human immunodeficiency virus sequences were pooled and ligated to *Sac*I arms of λgt WES.λB. The DNA was packaged by using the extract and protocol from Promega Biotech (Madison, WI). Plaques containing recombinant phage were located by the plaque filter hybridization.[20]

Restriction endonuclease mapping. Recombinant phage DNA was prepared as described.[18] DNA insert containing viral sequences were separated from λgt WES.λB arms by *Sac*I digestion and preparative agarose gel electrophoresis. Restriction endonuclease mapping was carried out with the partial digestion method of Smith and Birnstiel.[21]

RESULTS

Transfusion-associated AIDS patients and their blood donors. Previous studies in our laboratory revealed the presence of HIV in the blood of patients with transfusion-associated AIDS and their blood donors. All donor-recipient pairs had antibodies to HIV as determined by enzyme-linked immunosorbent assay and Western blot analysis.[15,16] Viruses isolated from four donor-recipient pairs were propagated in PHA-stimulated normal PBLs. The clinical conditions of the donor-recipient individuals is given in Table 1. In all cases, only one HIV-infected donor was found to be the source of

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Table 1. Findings in Investigations of Patients With Transfusion-Associated AIDS and Their Blood Donors

ID No. of Donor-Recipient Pair From Whom HTLV-III/LAV Was Isolated	Total No. of Donors	No. Donors With AIDS Risk Factor*/ No. Interviewed	No. Donors With HIV Antibody/ No. Tested	Interval From Transfusion to AIDS Onset in Recipient (mo)	Donor Health at Time of Viral Isolation
1	3	1/3	1/3	18	Healthy
2	9†	1/4	1/4	31	Healthy
3	34	1/30	1/26	18	Healthy‡
4	6	1/5	1/3	18	Healthy

Virus from the recipient was isolated at the time of AIDS onset.

*All donors with AIDS risk factor were homosexual men.

†Five donors were collected in Europe.

‡Subsequently developed AIDS.

the recipient's infection with virus. Interestingly, all the blood recipients had AIDS at the time of virus isolation, whereas all donors were asymptomatic during the time between blood donation and virus isolation.

Southern hybridization analysis of donor-recipient HIV proviral DNAs. High–molecular weight DNA isolated[17] from virus-infected cells was digested with restriction enzymes and analyzed by Southern hybridization to a molecularly cloned full-length HIV-Zr6 DNA. HIV-Zr6, isolated from an African AIDS patient, was cloned by using Hirt DNA extracted from the virus-infected cells. *Sac*I enzyme–digested DNA was used for cloning because this enzyme has cleavage sites only in the LTR sequences of viral DNA.[22] From our analyses involving a number of isolates from North America and Zaire (Anand et al, unpublished data) the 9-kb full-length probe hybridizes intensely to all isolates despite the restriction enzyme site differences. No detectable hybridization was found in uninfected cultures of the same PBL. Figure 1 shows the hybridization patterns observed with donor-recipient pair 1 proviral DNA cleaved with 14 different restriction enzymes. Donor DNA, cleaved with *Sac*I restriction enzyme, showed a faint hybridization at the 9-kb range in addition to ones at 5.5 kb and 3.5 kb. Further experiments by taking lesser amounts of high–molecular weight DNA in the restriction digests indicated that the 9-kb band was due to partial digestion and not to the presence of a second virus in the cells (data not shown). Also, restriction enzyme patterns show that only one predominant form of the virus was detected in a single donor or recipient.

Four donor-recipient pairs of viruses analyzed in our experiments exhibited varying degrees of restriction enzyme site polymorphism (Table 2). *Sac*I enzyme, which cleaves the viral DNA in long terminal repeat sequences of HIV,[5-7] generated different restriction fragments in three donor-recipient pairs: 1, 3, and 4. A characteristic feature of HIV-infected lymphocytes is the presence of considerable

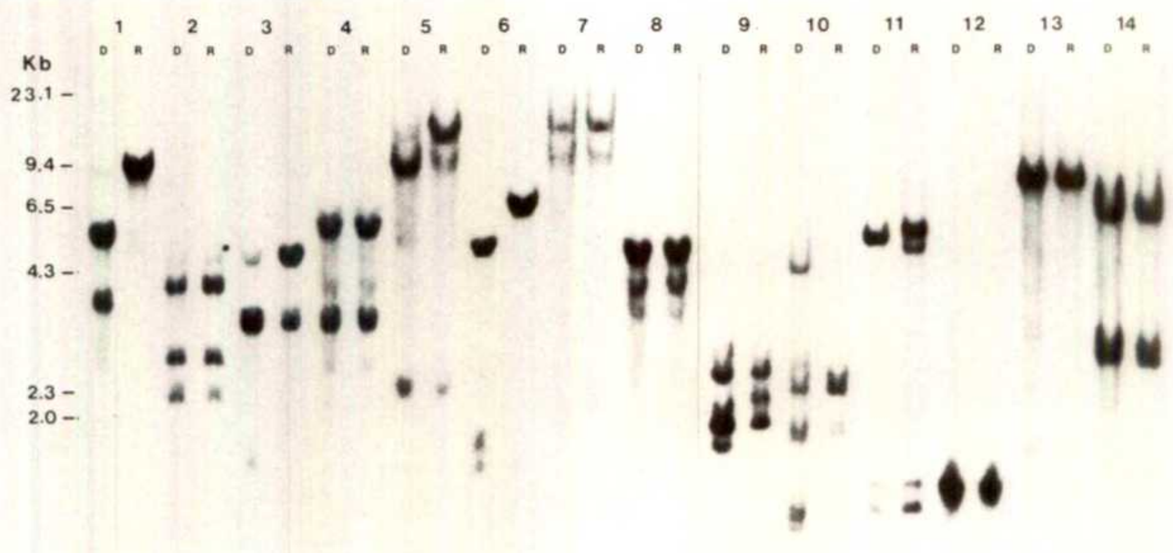

Fig 1. Southern blot analysis of HIV proviral DNA from donor-recipient pair 1. PBLs, separated by Ficoll-Hypaque technique and stimulated with PHA (10 µg/mL), were infected with HIV isolated from the respective donor-recipient pair as described.[15,16] Six days after infection, high–molecular weight DNA was extracted and digested with different restriction enzymes. Hybridization was carried out under standard conditions.[18] D, blood donor; R blood recipient; 1, *Sac*I; 2, *Kpn*I; 3, *Hind*III; 4, *Hinc*II; 5, *Eco*RI; 6, *Bgl*II; 7, *Bam*HI; 8, *Pst*I; 9, *Pvu*II; 10, *Sca*I; 11, *Hpa*II; 12, *Hph*I; 13, *Hha*I; 14, *Eco*RV. Full-length HIV-Zr6[22] fragment cloned in pUC18 plasmid was purified twice on low-melting agarose gel and used for nick-translation.

Table 2. Summary of Restriction Fragment Differences in Donor-Recipient Pairs of HIV

Virus Isolate	Restriction Enzyme									
	SacI	*KpnI*	*HindIII*	*HincII*	*EcoRI*	*BglII*	*BamHI*	*PstI*	*PvuII*	*ScaI*
Donor-recipient pair 1	+	–	+	–	+	+	–	–	+	+
Donor-recipient pair 2	–	–	+	–	–	–	–	–	+	–
Donor-recipient pair 3	+	–	–	–	+	–	–	–	+	–
Donor-recipient pair 4	+	+	+	+	–	+	–	–	+	–

Abbreviations: +, change in the restriction fragment length generated by the indicated enzyme between donor-recipient viral DNA; –, no change in restriction fragment length.

unintegrated linear and circular DNA in addition to the integrated proviral DNA.[5-7,12] To avoid the restriction site differences due to the different forms of DNA and to compare the internal restriction fragments, we have also tested with enzymes *BglII*, *HindIII*, *KpnI*, *PvuII* and *ScaI*, which cleave the genome at multiple sites. Table 2 lists the differences in the four pairs of viruses with regard to ten different restriction enzymes. Donor-recipient pairs 1 and 4 showed six restriction site differences, and donor-recipient pair 2 showed only two restriction site differences. Restriction analyses failed to reveal a specific genotypic pattern that distinguished isolates obtained from the AIDS patients and isolates obtained from the asymptomatically infected blood donors.

Molecular cloning of donor-recipient human immunodeficiency viral DNA. To determine more precisely the extent of genomic variation between donor-recipient viruses, we have molecularly cloned the viral genomes of donor-recipient pair 1 (Table 1). PHA-stimulated lymphocytes were infected at a high multiplicity with viruses derived from the donor-recipient pair. Unintegrated viral DNA extracted 72 hours after infection[19] was used as a substrate for constructing the recombinant DNA library, and positive clones were identified with the HIV-Zr6 probe. Representative clones containing donor and recipient viral genome were selected for detailed analysis. The physical maps of the cloned genomes are shown in Fig 2. Donor viral DNA had 17 unique restriction sites, and the recipient virus showed seven new restriction sites. Restriction enzymes *NcoI* and *SalI*, which cleave viral DNA only once, exhibited no alteration. On the other hand, changes were evident with restriction enzymes such as *HindIII*, *PvuII*, *BglII*, and *ScaI*, which cleave at multiple sites in the viral DNA. The restriction enzyme site differences between the donor and recipient viruses are noticeably clustered in the *env* and downstream *orf*-2 gene. Comparison of the physical maps of the donor-recipient viral DNA with that of HTLV-III and ARV indicated the presence of common restriction enzyme sites. In addition, donor-recipient viruses exhibited a number of differences from HTLV-III and ARV, especially with respect to the enzymes that cleave viral DNA less frequently. A primary nucleotide sequence comparison of donor-recipient viruses would be required to define these changes more precisely.

DISCUSSION

HIV has been shown to exhibit considerable genetic heterogeneity as determined by restriction enzyme cleavage patterns. Comparison of 18 different viral isolates by Wong-Staal et al[14] revealed that variation between isolates ranged from very minimal (one of 23 restriction enzyme sites) to considerable (16 of 31 enzyme sites different). Benn et al[23] have noted variation in ten isolates derived from different geographic locations. These differences in restriction enzyme

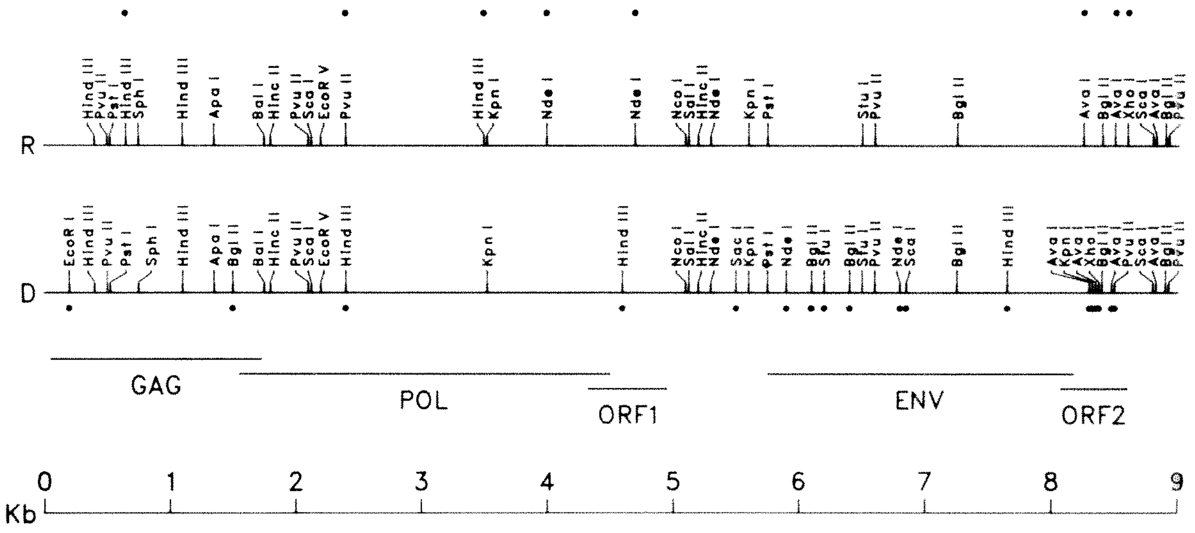

Fig 2. Restriction endonuclease maps of HIV from donor-recipient pair 1. The location of genes are indicated below the physical map. D, blood donor; R, blood recipient. ● indicates unique restriction sites present in the viral genome.

patterns could result from many kinds of genotypic differences[24] including: (*a*) one or more individual bases could differ, resulting in loss of the cleavage site or formation of a new one, and (*b*) insertion or deletion of blocks of sequences could also alter its size. Donor-recipient viruses not only share a number of common restriction enzyme sites but they also possess sites unique to each isolate. Our analysis of donor-integrated viral DNA suggests that there was only one kind of virus present in the donor's blood, which may be the predominant form at the time of collection. By using different HLA-unrelated donor lymphocytes, viruses with similar restriction pattern were repeatedly recovered, indicating a lack of genetically distinct viruses at the detectable level. However, we cannot rule out the possibility that blood donors harbor multiple viruses and that one may be able to grow better upon transmission into the recipient's body. In vitro lymphocyte cultures used for propagating the virus may have selected one form over the other. This possibility is less likely because PBLs seem to have less selection bias for virus growth in comparison with the established human T cells in culture.

Whether the changes in the genomic sequences result in the changes of the translational products encoded by the virus is a very important question. Neel et al[25] suggested that electrophoresis should detect about one third of all nucleotide substitutions in the coding sequences because of altered molecular charge of polypeptides due to amino acid substitutions. There are changes in the *gag* and *env* gene proteins encoded by donor-recipient viral genomes (data not shown) similar to the data reported for nine independent HIV isolates.[26] The differences in the apparent molecular weight of proteins may be due to substitutions in amino acid sequence and deletions as well. The changes in *env* gene protein may have significant implications because of its role in recognition of receptor and tropism.[11]

Molecular cloning of AIDS viruses from donor-recipient individuals enabled us to analyze the similarities and differences in detail. The differences between donor-recipient viruses are most prevalent in the *env* and *orf*-2 gene. It should be mentioned that a span of 18 to 31 months separate donor-recipient viruses. Noticeably, the extent of changes in the recipient virus does not correlate with the time elapsed from transfusion in the recipient because donor-recipient pair 2 showed minimal changes. Since the spontaneous mutation rate is much lower in humans, a special hypermutation mechanism may have to be invoked for the differences seen with donor-recipient viruses. Sequence analyses of different HIV isolates indicate that point mutation, presumably due to misincorporation of bases and rearrangements (reduplication, inversions, deletions, etc) contribute to the observed genetic variation between isolates.[27,28] Also, nucleotide variation is not consistent over the whole genome. The genes encoding the internal virion proteins *gag* and *pol* are considerably more conserved than *env*. Such a picture also emerges from the restriction enzyme data of the donor-recipient isolates and other independent isolates.[23] The underlying mechanism of such variation is not known. The error-prone nature of virus-encoded reverse transcriptase enzyme may partly be responsible for such variation.[28] Studies concerning the retrovirus point mutation rate with Rous sarcoma virus indicate a frequency on the order of 3×10^{-4} bases per passage of virus.[28] Hahn et al[29] reported a higher mutation rate for HIV. Studies on viruses recovered from molecularly cloned HIV proviral DNA using an animal model system would be useful to quantitate the mutation rate in HIV.

Wong-Staal and coworkers[14] reported that AIDS virus does not accumulate genetic changes in cell culture. The changes observed with different AIDS viruses, including the donor-recipient pair, may reflect the selective pressure of the immunologic mechanisms existing in different hosts. The changes in HIV may be similar to changes observed with visna virus[30,31] and equine infectious anemia virus (EIAV),[32] members of the lentivirus family. Both visna virus and EIAV undergo changes in their envelope glycoproteins during progression of infection. It has been suggested that such changes allow these viruses to evade host immune surveillance mechanisms. Whether the emergence of antigenic variants plays any role in the pathogenesis[33,34] of these diseases or in the pathogenesis of AIDS and related human diseases remains to be proved. In all four of the donor-recipient pairs studied, the recipients developed AIDS before their donors, and in three cases, the donors have so far remained asymptomatic carriers. The viral genotype, host immune surveillance mechanisms,[35] and/or other factors may contribute to the development of disease in the recipients. Recently, Hahn et al[29] reported genetic changes in HIV isolated sequentially from persistently infected individuals. The data generated on transfusion-associated AIDS viruses further attest the changing nature of HIV in vivo. It will be interesting to know whether HIV does change more rapidly when passing from one person to another than when multiplying in a single person. The elucidation of this should await further studies.

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The Effects of GM-CSF and G-CSF in Promoting Growth of Clonogenic Cells in Acute Myeloblastic Leukemia

By Edo Vellenga, Diane C. Young, Katherine Wagner, Donald Wiper, Diana Ostapovicz, and James D. Griffin

A small subset of leukemic cells from most patients with acute myeloblastic leukemia (AML) have properties of stem cells and can be assayed by colony formation in agar or methylcellulose. Colony formation generally requires the addition of exogenous growth factors, but the exact factors required are incompletely defined. The AML colony-promoting activities of two recombinant human colony-stimulating factors (GM-CSF and G-CSF) were investigated by using blasts from 48 patients with AML. In nine cases, no colonies formed with either CSF. In seven cases colonies formed only in response to G-CSF and in 11 cases only in response to GM-CSF. In 21 cases colonies formed in response to either GM-CSF or G-CSF, and in 12 of these cases there was an additive effect between the two CSFs in determining maximum colony size. For cases responding to both GM- and G-CSF, the total number of colonies formed in response to the combination of both CSFs was almost always less than additive compared with the number of colonies formed in response to the individual CSFs. Further, the AML-CFU responding to either GM-CSF or G-CSF could not be distinguished by surface markers or by the cytochemical staining pattern of the colonies. These results suggest that there is considerable overlap between the GM-CSF– and G-CSF–responsive AML-CFU subpopulations in most cases. For five of seven cases, the combination of GM-CSF and G-CSF could replace a leukocyte feeder layer in providing maximum growth stimulation. These results indicate that GM-CSF and G-CSF are active growth factors for AML cells and are frequently additive in promoting maximum colony size.

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PROLIFERATION of acute myeloblastic leukemia (AML) cells in vitro is limited in most cases to a small subset of blasts that have several properties characteristic of active stem cells: self-renewal potential, high thymidine suicide index, and the capacity to undergo at least partial differentiation.[1] Growth of these colony-forming cells (AML-CFU) from most patients requires the addition of colony-stimulating factors (CSFs) that have been traditionally supplied by lectin-activated normal blood cells[2] or media from various tumor cell lines.[3,4] It has been difficult to identify the exact factors that promote growth of AML cells in these complex media since multiple hematopoietic growth factors are present.[5] Our laboratory[6] and others[7,8] have previously studied the effects of recombinant human GM-CSF and shown that this CSF actively promotes growth of AML-CFU. In fact, for many cases of AML, GM-CSF could completely replace standard conditioned media.[6] In other cases, however, growth with GM-CSF alone was suboptimal, and in some cases no growth of AML-CFU was observed at all.[6,8]

The genes for other human CSFs have now been cloned[9-11] and purified recombinant proteins produced. In an effort to further identify the growth factors required for proliferation of AML-CFU and to compare these requirements with those of normal myeloid progenitor cells, we have investigated the effects of recombinant G-CSF and GM-CSF on the growth of AML-CFU and normal CFU-GM by using several culture systems. The results indicate that, although GM-CSF is the most active single factor, G-CSF also promotes growth of AML-CFU from many patients with AML and is additive with GM-CSF in many cases.

MATERIALS AND METHODS

Leukemic cells. Peripheral blood or bone marrow aspirate samples were obtained at diagnosis from 48 adult patients with AML. All patients gave informed consent under guidelines approved by the Institutional Review Board. The diagnosis of AML was established by morphology, cytochemical staining, and surface marker analysis using a panel of antimyeloid monoclonal antibodies.[12] Leukemic cells were recovered by Ficoll-diatrizoate density gradient sedimentation, and mononuclear cells were cryopreserved in 10% dimethylsulfoxide in the vapor phase of liquid nitrogen until use. Peripheral blood was used whenever possible to minimize the possibility of "contamination" of AML-CFU with normal CFU-GM because of the very low numbers of CFU-GM found in normal blood.[6] After thawing, samples were required to have a viability of greater than 90% and to contain more than 80% blasts. T lymphocytes were removed by rosetting with sheep erythrocytes, and adherent monocytes were removed by plastic adherence (one hour at 37°C). Monocyte contamination of the leukemic samples before the adherence procedure was low. Fifty-three percent of the samples contained less than 4% monocytes, detected with the monoclonal antibody MY4. In 28% of the samples more than 20% of MY4-positive cells were observed. However, these cells were felt to be primarily leukemic on the basis of blast morphology.

Normal bone marrow. Normal marrow was aspirated from volunteers; mononuclear cells were isolated by Ficoll gradients and generally cryopreserved prior to culture. All donors gave informed consent. BALB/c mouse marrow was removed from femurs.

Clonogenic assays. 1. A double layer agar assay was performed in triplicate by modification of the method of Pike and Robinson.[13] Sources of CSF were incorporated in a 0.5% agar (Noble agar, Difco, Detroit) underlayer (0.5 mL). Target leukemic cells (1 to 5×10^4/0.5 mL) were placed in a 0.3% agar overlayer. The medium was Iscove's modified Dulbecco's minimal essential medium (GIB-

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CO, Long Island, NY) containing 20% fetal bovine serum further supplemented with glutamine. The cell number was adjusted to generate fewer than 100 colonies/culture. At seven to ten days of culture, the agar overlayers were removed from the underlayers by agitation and dried onto glass slides under filter paper. Dried agar overlayers were fixed in acetone-citrate-methanol and colonies stained in situ for chloroacetate esterase and alpha-naphthyl acetate esterase activities to identify cells of granulocyte and monocyte lineage.[3,6] Colonies were defined as aggregates of 20 or more cells.[2] Normal CFU-GM were assayed as previously described by using the double-layer agar assay system.[13]

2. The phytohemagglutinin–leukocyte feeder assay (PHA-LFS) was performed as described by Löwenberg et al.[14] The agar underlayer contained irradiated normal peripheral blood leukocytes. In some experiments the feeder leukocytes in the underlayer were omitted and replaced by recombinant human CSFs. Colonies (greater than 50 cells) and clusters (8 to 50 cells) were counted on an inverted microscope on days 7 to 10.[14] Colonies from representative cultures from each patient were pooled and the percentage of T lymphocytes determined by E-rosetting.

CSFs. Recombinant human CSFs were obtained from Drs Steven Clark and Gordon Wong, Genetics Institute, Cambridge, MA. The CSFs were obtained as media conditioned by COS cells transfected with the P91023(B) expression vector containing full-length cDNA clones of each of the human genes.[15] Medium conditioned by COS cells transfected with the same vector but not containing any CSF gene was used as a control (mock-CSF) and contained no detectable CSF activity for normal human or mouse CFU-GM or AML-CFU. To supply an excess of each CSF for AML-CFU experiments, each CSF was titered for its ability to promote maximal growth of normal marrow CFU-GM. A concentration of at least twofold more than what was required to generate maximum CFU-GM was used to stimulate AML-CFU. In one experiment, recombinant erythropoietin (2 U/mL, Genetics Institute) was added to methylcellulose cultures.

Determining the surface antigen phenotype of AML-CFU. Since in many cases colonies were formed in response to more than one type of CSF, the surface antigen phenotype of clonogenic cells was determined in an effort to determine whether the same or different populations of clonogenic cells were proliferating to each factor. Briefly, the surface antigen phenotype was determined by complement lysis with monoclonal antibodies anti–HLA-DR, -MY9, -PM-81, and -AML-2-23.[16] Following treatment with antibody and complement, the residual number of viable clonogenic cells was assayed in agar culture and compared with the number present after treatment with a control monoclonal antibody and complement. The lot of C′ used in these studies was preselected for low toxicity to marrow progenitor cells.

Surface antigen analysis of AML cells. Aliquots of AML cells were incubated with monoclonal antibodies detecting HLA-DR, MY4, MY7, MY9, or glycophorin A for 30 minutes at 4°C, washed twice, further incubated with fluoresceinated goat antimouse Ig (Coulter Immunology, Hialeah, FL) for 30 minutes at 4°C, and analyzed by flow cytometry.[12] The presence of 20% of cells more fluorescent than the background was considered positive.

RESULTS

Recombinant CSF titers. The titers of recombinant CSF-containing conditioned media were determined by assaying the ability of each CSF to support a specific type of normal CFU-GM. GM-CSF supported growth of human granulocyte and monocyte colonies; G-CSF supported growth of human and murine granulocyte colonies (Table 1). Based on this information, AML-CFU experiments were performed with concentrations of GM-CSF of 1:500; G-CSF was used at 1:1,000.

Table 1. Effects of Recombinant Human CSFs on Normal Marrow CFU-GM

		Day 7 CFU-GM/5 × 10^4 Cells†			
		Human		Murine	
CSF	Titer*	Granulocyte	Monocyte	Granulocyte	Monocyte
rGM-CSF	1:500	99	29	1	1
	1:1,000	103	27	0	0
	1:2,000	64	25	0	0
	1:4,000	18	6	0	1
rG-CSF	1:500	123	3	73	9
	1:1,000	131	6	75	8
	1:2,000	129	1	72	7
	1:4,000	31	3	61	6
Mock-CSF	1:500	2	1	1	0
Control medium		1	2	0	2

*Recombinant human CSFs used as media conditioned by COS cells transfected with plasmids containing CSF cDNAs. Titer is the final dilution of stock conditioned medium. Mock-CSF is medium conditioned by COS cells transfected with vector only.

†Target cells are human or mouse (BALB/c) light-density, nonadherent marrow cells grown in the overlayer of a double-layer agar system. All CSFs were incorporated in the lower layer. Clusters of greater than eight cells were enumerated on day 7 and expressed as the mean of quadruplicate cultures. Standard errors of the mean were less than ±20% in each case.

AML cases. Cells from 48 randomly selected AML patients were tested for response to G-CSF, GM-CSF, and the combination of both CSFs in agar culture. No colony growth was observed in nine cases (19%) with any of the CSFs tested (data not shown). In nine cases there was autonomous formation in the absence of any CSFs of more than 20 colonies per culture (range, 28 to 705 AML-CFU/5 × 10^4 cells). In 81%, AML-CFU growth was observed in response to one or both CSFs, defined as more than ten AML-CFU/5 × 10^4 plated cells if there was no autonomous growth or more than a twofold increase in the number of AML-CFU compared with the control medium if there was autonomous proliferation (Table 2). Fifteen percent of cases responded to G-CSF only (cases 1 to 7, Table 2), 23% of cases responded to GM-CSF only (cases 8 to 18, Table 2), and 44% responded to either G-CSF or GM-CSF (cases 19 to 39, Table 2). In two thirds of these latter cases higher colony numbers were observed with GM-CSF in comparison to G-CSF, whereas in 14% the response to G-CSF was more pronounced than the response to GM-CSF.

In 15 patients (Table 2) the combined effects on colony size (measured by colony diameter) of GM-CSF and G-CSF were greater than the maximum effects observed with either factor alone by twofold or more. In separate experiments with six of these cases, the concentration of GM-CSF was increased tenfold over the concentration used in Table 2 without further increasing colony number or size. The addition of G-CSF (1:1,000) remained additive in causing an increase in colony size (data not shown).

Table 2. Effects of CSFs on Growth of Clonogenic AML Cells

		AML-CFU/5 × 10⁴ Cells*				
Case	PBL/BM	Medium Control	G-CSF, 1:1,000	GM-CSF, 1:500	G-CSF + GM-CSF	Additive Effects on Colony Size with G- and GM-CSF†
Response to G-CSF only						
1	PBL	28	69	32	75	+
2	PBL	0	870	2	855	−
3	BM	10	196	12	255	+
4	BM	1	49	3	51	−
5	BM	4	18	6	18	−
6	PBL	0	24	0	26	−
7	PBL	8	313	9	321	−
Response to GM-CSF only						
8	PBL	0	1	8	12	+
9	BM	0	3	56	57	−
10	PBL	0	2	78	81	−
11	PBL	85	87	95‡	98	−
12	PBL	0	4	78	75	−
13	BM	1	1	61	59	−
14	PBL	2	4	173	173	−
15	BM	1	1	48	46	−
16	PBL	0	1	22	29	−
17	BM	0	0	62	71	−
18	BM	0	0	215	222	−
Response to both G-CSF and GM-CSF						
19	BM	0	46	69	110	+
20	PBL	225	235‡	451	479	+
21	PBL	167	217‡	231‡	261	+
22	PBL	76	191	164	207	+
23	PBL	2	21	47	47	−
24	PBL	62	152	211	231	+
25	PBL	0	12	17	19	−
26	PBL	0	42	82	105	+
27	BM	19	173	140	257	+
28	PBL	0	109	31	143	+
29	PBL	0	5	17	18	−
30	PBL	12	34	62	145	+
31	BM	28	74‡	79	81	+
32	PBL	13	61	69	79	−
33	PBL	36	316	497	502	−
34	BM	3	32	71	69	−
35	PBL	0	165	329	341	−
36	PBL	705	801‡	821‡	1,150‡	+
37	BM	0	38	32	40	−
38	BM	0	38	226	911	+
39	BM	0	46	211	207	−

Abbreviations: PBL, peripheral blood; BM, bone marrow.

*AML-CFU were assayed in agar as described in Materials and Methods. The results are presented as the mean of triplicate cultures. The standard errors were less than ±25% in all cases.

†An additive effect was defined as an increase in average colony diameter by twofold or greater with the combination of G-CSF plus GM-CSF compared with colonies grown in response to either factor alone.

‡For cases in which there was significant autonomous colony formation, an increase in colony diameter by twofold or more in response to a CSF was also considered a response.

In most cases, the additive effects of the combination of GM-CSF and G-CSF did not apply to colony number. In only three of 15 cases did the increase in colony number exceed 50% (59%, 133%, and 301%). These results indicate that both G-CSF and GM-CSF are active growth factors for supporting AML-CFU and that G-CSF and GM-CSF are additive in determining the maximum colony size in many cases. The distribution of CSF response type compared with whether or not there was autonomous growth is summarized in Fig 1.

Surface antigen phenotype of AML-CFU responding to GM-CSF and G-CSF. The results in Table 2 show that in many cases of AML there are AML-CFU that can respond to both G-CSF and GM-CSF. In those cases further efforts

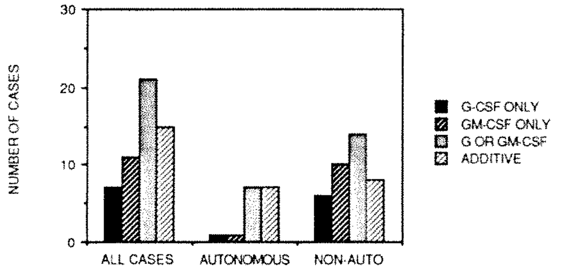

Fig 1. Distribution of cases by CSF response and autonomous growth of clonogenic cells. The additive effect was defined as an increase in colony diameter twofold greater than the maximum effects observed with either factor alone.

were undertaken to determine whether two populations of clonogenic cells were present or only a single population. Ten such cases were selected, myeloid blast cells were lysed with antimyeloid monoclonal antibodies and complement, and surviving clonogenic cells were assayed in cultures containing either GM-CSF or G-CSF (Table 3). The "phenotype" of the clonogenic cells proliferating in response to each factor was found to be closely comparable.

Colonies from six of these ten patients were also stained in situ for expression of the granulocytic marker chloroacetate esterase (CAE) and the monocytic marker alpha-naphthyl acetate esterase (ANAE). As previously observed by our laboratory[16] and in contrast to the heterogeneous staining pattern observed in normal marrow, AML colonies from individual cases tend to be homogeneous: either CAE+ ANAE−, CAE− ANAE+, or CAE+ ANAE+ (both enzyme activities detected within single cells). Homogeneity of the staining pattern was also observed in these cases (Table 3). The staining pattern in individual cases was the same when the colonies were stimulated by either GM-CSF or G-CSF. It is noteworthy that in three cases G-CSF promoted the growth of ANAE+ CAE− colonies, a situation not observed in normal marrow (Table 1).

Table 3. Phenotype of AML-CFU Proliferating in Response to GM-CSF or G-CSF

		Percentage of AML-CFU Expressing Antigen*				Colony Cytochemistry†	
Case No.	CSF	HLA-DR	MY9	PM-81	AML-2-23	CAE	ANAE
19	GM	71	9	70	66	−	+
	G	52	0	56	58	−	+
21	GM	99	72	68	72	+	+
	G	99	75	74	77	+	+
22	GM	82	52	91	95	−	+
	G	80	48	91	90	−	+
23	GM	70	95	93	98	NT	NT
	G	79	100	79	90	NT	NT
24	GM	85	93	31	0	+	−
	G	84	97	10	0	+	−
27	GM	87	50	91	90	NT	NT
	G	79	71	NT	86	NT	NT
28	GM	63	45	57	10	+	−
	G	65	51	60	15	+	−
32	GM	98	100	98	38	NT	NT
	G	100	100	100	20	NT	NT
33	GM	59	88	76	52	NT	NT
	G	54	88	69	62	NT	NT
34	GM	95	5	52	0	−	+
	G	93	0	59	17	−	+

*Leukemic cells were incubated with monoclonal antibodies and complement and residual viable AML-CFU determined by assay in agar. The percentage of AML-CFU expressing each antigen was taken as the percentage of AML-CFU lysed by antibody and C′ compared with C′ alone.

†Colonies were stained in situ for CAE activity and ANAE activity. +, 95% colonies staining; −, 5% colonies staining; NT, not tested.

Correlation of monocytic and erythroid phenotype with response to CSFs. The surface antigen phenotype of the whole leukemic cell population was determined in 39 cases by using anti−HLA-DR, -MY4 (monocyte), -MY7 (monocyte-granulocyte/CFU-GM), -MY9 (monocyte/progenitor cell), -T11 (T cell) and −glycophorin A (clone 39, erythroid) monoclonal antibodies. None of the cases had more than 2% T11+ cells. The 39 cases were grouped by CSF response (see Table 2), and the percentage of cases in each group expressing each surface antigen is shown in Table 4. Four of the five cases that expressed glycophorin A were in the nongrowing group. Eight cases that had autonomous growth had a phenotype similar to those responding to G- or GM-CSF, except that expression of MY7 was more common. These results suggest that erythroleukemias (glycophorin A−positive) tend not to respond to the CSFs tested in the culture system used. Cells from two of the glycophorin A−positive cases were retested for colony formation in the presence of erythropoietin (2 U/mL) in the presence and absence of GM-CSF or G-CSF. No colony formation was observed.

Identification of other CSFs that promote growth of AML-CFU. PHA-LFS has been shown to be a particularly effective culture system in promoting the growth of colonies of maximum size from the maximum number of patients.[14] We therefore sought to determine whether the previously undefined CSFs of the PHA-LFS could be partially or completely replaced by G-, GM-CSF, or a combination of the two. The distinction between clusters and colonies as originally defined by Löwenberg was retained.[14] In one case (20, Table 5) colony growth was more than twofold better in the PHA-LFS than with the combination of G- and GM-CSF. In the other cases, the difference was insignificant or

Table 4. Correlation of Surface Marker Expression With Response to CSFs

	Percentage of Cases Expressing Antigen by CSF Response				
Surface Antigen*	G-CSF†	GM-CSF†	G- or GM-CSF	No Growth	Autonomous
N	5	9	16	9	8
HLA-DR	60	89	94	56	100
MY4	0	56	13	22	13
MY7	60	89	38	33	75
MY9	80	89	50	22	89
Glycophorin A	0	0	6	44	0

*The surface antigen expression of the total leukemic cell population was determined by immunofluorescence using flow cytometry. The presence of greater than 20% stained cells was considered positive.

†These cases responded only to G-CSF or GM-CSF (see Table 1).

Table 5. A Comparison of Recombinant CSFs With the PHA–Leukocyte Feeder Culture System

	AML-CFU/10^5 Cells (Colonies/Clusters)*					Responses to CSF in Agar	
Case No.	Control	Irradiated Leukocytes	G-CSF	GM-CSF	G- + GM-CSF	G-CSF	GM-CSF
20	2/56	92/234	13/240	—	34/92	+	+
21	0/10	20/72	4/27	10/230	15/159	+	+
22	32/1,400	50/1,500	50/1,330	30/1,600	43/1,300	+	+
27	3/20	51/103	10/50	32/84	34/100	+	+
28	0/0	6/14	0/6	0/10	0/17	+	+
29	0/0	7/19	1/9	5/25	10/44	+	+
33	0/29	16/147	19/146	45/170	48/126	+	+

*AML-CFU were assayed as described by Löwenberg[14] except that in some cases the agar layer contained recombinant CSFs rather than irradiated leukocytes. The liquid overlayers of all cultures contained PHA. Clusters and colonies were counted on days 7 to 10. The results are the mean of duplicate cultures. Final concentrations of G-CSF were 1:1,000 and for GM-CSF, 1:500.

the recombinant CSF combination was better than the PHA-LFS.

DISCUSSION

The purpose of this study was to further define the growth factors required by clonogenic AML cells for in vitro proliferation. The results confirm an earlier study in which it was shown that GM-CSF was an active growth factor for AML-CFU, but probably not the only such factor.[6,8] In this study the effects of recombinant GM-CSF and G-CSF were tested and compared with the effects of the combination of these two CSFs. GM-CSF was the most active of the factors, followed closely by G-CSF. Overall, 67% of cases contained AML-CFU responsive to GM-CSF, 59% of cases contained AML-CFU responsive to G-CSF, and 44% of cases contained AML-CFU responsive to both.

The combination of G-CSF and GM-CSF was additive in determining maximum colony size in 15 cases compared with either CSF alone. This could occur in several ways. The initial AML-CFU could be responsive to both CSFs. Alternatively, the initial AML-CFU could be responsive only to G- or GM-CSF, and the interaction of that CSF with its receptor could induce expression of receptors for the other CSF. Future studies with purified AML-CFU may help resolve this question. The effects of the combined CSFs on the total colony number was variable. In seven of the 21 cases responding to both CSFs individually, the total colony number formed in response to the combination of CSFs increased to approximately the sum of the G-CSF colonies plus the GM-CSF colonies. In the remaining 14 cases, little or no increase in colony number was observed. These results suggest that in some cases the GM-CSF–responsive and G-CSF–responsive AML-CFU are partly or completely distinct, whereas in other cases the same clonogenic cells appear to respond to both growth factors. Surface antigen phenotype analysis of ten cases failed to distinguish G-CSF– from GM-CSF–responsive AML-CFU, further suggesting that these AML-CFU subsets are closely related in many cases.

The results in this and our previous report[6] indicate that GM- and G-CSF can provide near-optimum growth factor activity for in vitro proliferation of AML-CFU. There may well, however, be yet identified factors that are important for in vitro or in vivo growth. The results presented in Table 5 indicate that for most patients studied either GM-CSF alone or the combination of G-CSF and GM-CSF can substitute for activated leukocytes in the PHA-LFS. However, one case was identified in which the colony number was greater in the PHA-LFS, thus suggesting that yet another CSF such as human interleukin 3[17] may be produced by stimulated leukocytes. It is noteworthy that GM-, G-, and M-CSF can all be produced by normal blood leukocytes with appropriate stimulation. GM-CSF is secreted by activated T cells[15] and G-CSF (Young D, Rambaldi A, Griffin JD, unpublished observations) and M-CSF by activated monocytes.[18]

Several conclusions can be drawn from these results about the substructure of the AML stem cell compartment. First, AML-CFU from most patients require CSFs for in vitro growth and respond to the same concentrations of GM-CSF and G-CSF that also effectively promote growth of normal CFU-GM. In contrast to normal CFU-GM, however, AML-CFU from 15% of patients responded only to G-CSF, and AML-CFU from 23% responded only to GM-CSF. This observation defines a new level of heterogeneity among AML patients. Second, in cases containing both GM- and G-CSF–responsive AML-CFU, it is likely that the populations of clonogenic cells responding to G-CSF and GM-CSF overlap to a considerable degree in some but not all cases. Third, "aberrent" response to CSF can also be observed in that monocytic colonies (ANAE+) are formed in response to G-CSF.

As in our previous studies,[6] "autonomous" growth of clonogenic cells was observed in several cases. In each case in which there was autonomous growth, the size and/or number of colonies could be increased by the addition of one or more CSFs (seven of nine cases with autonomous growth responded to both GM-CSF and G-CSF).

Our laboratory has previously studied three cases of AML in which autonomous proliferation of leukemic cells occurred in agar culture.[19] Cells from two of these cases were found to express the GM-CSF gene constitutively, and both cases secreted biologically active GM-CSF. In an additional study it was shown that expression of the GM-CSF gene is not restricted to cases that can grow autonomously in agar and, in fact, expression can be observed in up to 50% of all cases of AML.[20] It is possible that production of GM-CSF by leukemic cells in vivo can substitute for CSFs from normal cellular sources such as T cells and endothelial cells, or they can augment the supply of CSF to the leukemic cells. Also, there are likely to be selective pressures in vivo for AML cells to develop mechanisms to use CSF in novel ways or to overcome the requirement for CSF completely. A few cases of CSF-independent growth have been observed in AML,[19] and the possibility that AML cells can express CSF-like proteins in their membranes may represent a unique solution for the problem of supplying adequate amounts of growth factor to a rapidly expanding tumor cell population.[21]

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Spectrin Oxidation Correlates With Membrane Vesiculation in Stored RBCs

By G.M. Wagner, D.T-Y. Chiu, J-H. Qju, R.H. Heath, and B.H. Lubin

An increase in spectrin oxidation in a variety of erythrocytes displaying a tendency to vesiculate has been previously described. To explore this relationship in more detail, we have studied blood stored in citrate-phosphate-dextrose-adenine under blood bank conditions because, in this system, vesiculation occurs slowly. Vesiculation was quantitated by measuring acetylcholinesterase release, and the extent of spectrin oxidation was detected by using thiol-disulfide exchange chromatography. A strong correlation ($r = .92$) was found between the extent of spectrin oxidation and vesiculation when blood from five donors was analyzed at weekly intervals during storage. This strongly suggests that spectrin oxidation plays a role in the formation of spectrin-free vesicles, thereby limiting the shelf life of stored blood.

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THE MECHANISM BY which the RBC membrane vesiculates is of particular importance in blood banking since posttransfusion viability of RBC appears to be limited by reduction in cell surface area.[1-3] Shedding of spectrin-free vesicles from echinocytic erythrocytes leads to the formation of dense, poorly deformable spherocytes that are quickly removed from the circulation.[2,3] In the past, several factors including cellular levels of adenosine triphosphate (ATP),[4-6] the behavior of the calcium ion,[7,8] and the structure and function of membrane proteins[9,10] have been extensively analyzed in stored RBC, but none has been found clearly to correlate with the development of spherocytes. Recently, Wolfe et al have demonstrated that a defect in the spectrin-actin-protein 4.1 interaction developed during storage and that normal protein interactions were restored with reducing agents.[11]

We have previously examined several red cell disorders (sickle cell anemia, hemoglobin H disease, and hereditary spherocytosis) and in vitro manipulations (ATP depletion, Ca^{+2} loading, and diamide treatment) in which vesiculation occurs.[12] In each case, membrane protein oxidation could be detected[12] by thiol-disulfide exchange chromatography.[13] Therefore, as will be described, we quantitated spectrin oxidation as RBC slowly vesiculate during storage in citrate-phosphate-dextrose-adenine (CPD-A). Under these conditions, there was a striking correlation between spectrin oxidation and vesiculation.

MATERIALS AND METHODS

Determination of the Extent of Vesiculation

Acetylcholinesterase determination. The activity of AChE was assayed as described by Ellman et al.[14] This assay was used as a measure of extent of red cell vesiculation as initially outlined by Ott et al.[15]

Measurement of phospholipid release. Vesiculation of washed fresh, normal erythrocytes (hematocrit, 20%) was induced by incubation in 10 mmol/L Tris-buffered saline (TBS), pH 7.4, containing 1 mmol/L $CaCl_2$ and 2 μmol/L A23187, the calcium ionophore. This is a slight modification of the method of Allan and Thomas[16] using a lower concentration of ionophore to slow the rate of vesiculation. To stop the vesiculation process, samples were removed at four-minute intervals and pipetted into 100 mmol/L EDTA to yield a final concentration of 3 mmol/L. The red cell count of the cell suspension was determined in a Coulter counter (Coulter Electronics, Hialeah, FL). The phospholipids in 200 μL of cell suspension were extracted by the method of Rose and Oklander,[17] and the phospholipids in 1 mL of supernatant were extracted by the method of Bligh and Dyer.[18] Inorganic phosphorus in these samples was measured after sulfuric acid hydrolysis.[19]

ATP Measurement

ATP was extracted from RBC with perchloric acid by the method of Beutler[20] and was quantitated by using a commercially available luciferin/luciferase assay (Packard, Downers Grove, IL).

Osmotic Gradient Ektacytometry

Osmotic gradient ektacytometry was performed by the method of Clark et al.[21] This technique measures the deformability index (DI) of erythrocytes over a continuous osmotic gradient.

Thiol-Disulfide Exchange Chromatography

Thiol-disulfide exchange chromatography was performed with the method of Rank et al.[13] Theoretically, when this technique is used, proteins with no available sulfhydryl groups (filtrate fraction) do not bind to the resin and can be separated from proteins containing sulfhydryl groups available for binding. The bound proteins can be eluted with the reducing agent, β-mercaptoethanol (gel fraction). When the original solubilized membranes, the filtrate (oxidized), and the gel (reduced) fractions are analyzed by reducing Laemmli sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis,[22] it is apparent that after chromatography band 7 is present only in the filtrate fraction (Fig 1). Therefore, the extent of spectrin oxidation in a given sample can be expressed in terms of the spectrin:band 7 ratio determined by densitometric scanning of the gel. The ratio of band 7 to band 3 in the original membranes not subjected to chromatography was determined to assess the stability of band 7 during storage.

Determination of the Sulfhydryl Content of the Oxidized Fraction by Using Amino Acid Analysis

Ghosts were prepared from erythrocytes that had been incubated at 37°C for 48 hours in TBS, pH 7.4, containing penicillin and streptomycin, to induce protein oxidation by ATP depletion.[23] Thiol-disulfide chromatography was performed on these ghosts and the

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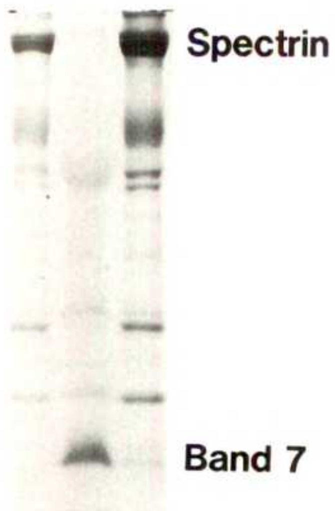

Fig 1. Laemmli SDS-polyacrylamide gel electrophoresis of the thiol-disulfide gel (reduced) fraction (lane 1), filtrate (oxidized) fraction (lane 2), and original unchromatographed ghost membranes (lane 3). Note that band 7 is found in the oxidized but not the reduced fraction.

oxidized fraction (consisting predominantly of spectrin, hemoglobin, and proteolytic fragments) obtained. The free sulfhydryl content of the oxidized proteins was measured by labeling them with iodoacetic acid, a reagent that reacts with free sulfhydryl groups to form carboxymethyl cysteine.[24] Initially, the oxidized fraction was divided into two aliquots, one of which was immediately reduced in 2 mmol/L dithiotheitol (DTT). The oxidized aliquot was dialyzed overnight at room temperature against 8 mmol/L urea, 0.1 mol/L Tris, pH 8.5; the reduced aliquot was dialyzed against the same buffer containing 2 mmol/L DTT. After dialysis the samples were placed in screw top tubes, flushed with N_2, sealed, and incubated at 30°C for 30 minutes. Then 0.5 mol/L iodoacetic acid was added to a final concentration of 10 mmol/L and the samples incubated in the dark for an additional 30 minutes at 30°C. The reaction was terminated by adding one drop of β-mercaptoethanol. The fractions were dialyzed overnight against 2 mol/L urea, 50 mmol/L Tris, pH 8.5, at 4°C and then for 48 hours against water. The samples were acid hydrolyzed, and amino acid analysis was performed.[25]

Blood Storage

Four units of blood obtained from four normal donors and two units, drawn on separate occasions from a woman therapeutically phlebotomized for polycythemia secondary to chronic lung disease, were stored in CPD-A under standard blood bank conditions for 8 weeks. Samples were removed at weekly intervals for AChE determinations to quantitate the extent of vesiculation occurring during storage, ATP extraction, ektacytometry, and thiol-disulfide chromatography.

RESULTS

AChE Release Is Linearly Correlated With Phospholipid Release

The initial goal of these studies was to demonstrate the validity of the methods used to quantitate both vesiculation and spectrin oxidation. The extent of vesiculation during storage was quantitated by measuring the percentage of the total whole blood AChE that was present in the plasma. However, this percentage does not equal that of phospholipid released because AChE is concentrated in vesicles.[26] Because storage vesiculation takes place in lipid-rich plasma, determining the nature of the correlation between AChE release and phospholipid release was not feasible in this system. Therefore to examine this problem, we induced vesiculation in fresh washed erythrocytes by calcium loading and found that AChE release is linearly related to phospholipid release (Fig 2). The calcium-loading system was used in these experiments because maximum vesiculation could be achieved in 60 minutes, the vesiculation process could be halted instantly by the addition of EDTA, and most importantly, there was no exogenous phospholipid in the incubation buffer. A similar linear relationship between AChE and phospholipid release was found when vesiculation due to ATP depletion was studied (data not shown). Since such a relationship is present in two very different forms of the vesiculation process,[23] we feel that a linear relation between AChE release and phospholipid release during storage vesiculation is highly likely as well. Therefore in all storage experiments we measured AChE release to quantitate vesiculation.

Measurement of Spectrin Oxidation

To quantitate the extent of spectrin oxidation, we performed thiol-disulfide exchange chromatography on solubilized ghost membranes to separate oxidized and reduced proteins. The original ghost membranes and both column fractions were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. The extent of spectrin oxidation was quantitated by measuring the ratio of spectrin

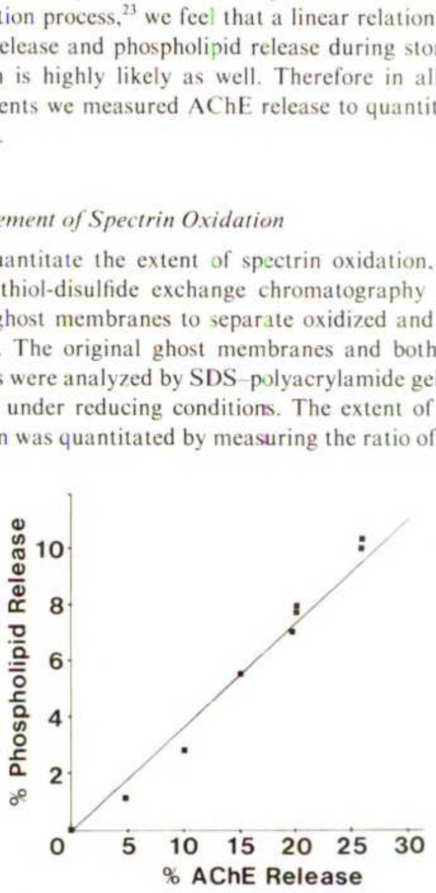

Fig 2. Percentage of AChE released during calcium loading v percentage of phospholipid release (a representative experiment).

to band 7 in the oxidized fraction. To ensure that changes in this ratio during storage were not due to changes in band 7, the band 7:band 3 ratio in the original membrane samples was measured. In all samples this ratio remained constant during storage (0.10 ± 0.02), thus indicating that there was no significant loss of band 7 and that changes in the spectrin:band 7 ratio were due to changes in the amount of oxidized spectrin.

Amino acid analysis experiments indicate that proteins in the filtrate fraction are approximately two-thirds oxidized. An aliquot of oxidized proteins treated with iodoacetic acid contained only 34.5% as much carboxymethyl cysteine as an aliquot reduced before treatment. The oxidized fraction analyzed in this way was a mixture of proteins, so the exact extent of spectrin oxidation cannot be stated. Nevertheless, it is reasonable to assume that the proteins in the oxidized fraction have been oxidized to a similar degree. Therefore, the thiol-disulfide chromatographic method allows us to quantitate spectrin that has been severely oxidized.

Vesiculation of Erythrocytes During Storage

We have previously demonstrated increased spectrin oxidation in a variety of conditions known to be associated with red cell vesiculation.[12] To examine this phenomenon in more detail we have selected an experimental system in which vesiculation occurs slowly, namely, in blood stored under blood bank conditions. Figure 3 shows AChE release during storage of units of whole blood from four different donors. There is individual variation in the rate and extent of vesiculation. This is consistent with previous observations that there are differences in the storage stability of blood from different donors.[2,3] An ektacytometer was used to determine the deformability of a "rapid" and a "slow" vesiculating unit before and after 6 weeks of storage. The deformability pattern of the rapid unit showed a decrease in maximal deformability during storage consistent with membrane loss, whereas the pattern of the slow unit was only slightly changed (Fig 4).

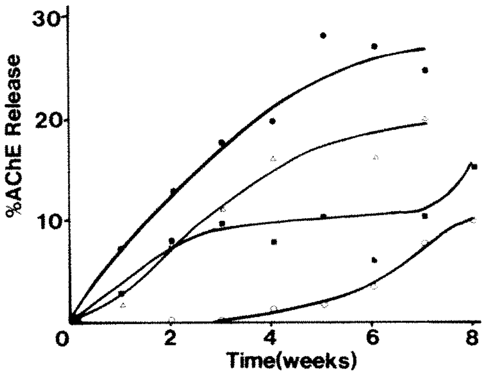

Fig 3. Percentage of AChE released from 4 units of stored RBC *v* time. Donor 1, ●—●; donor 2, △—△; donor 3, ■—■; donor 4, O—O. Note: Units from donor 1 were analyzed on two separate occasions and gave similar results each time. Blood from a fifth donor behaved in a manner virtually identical to donor 1, but for the sake of clarity, the data are not shown.

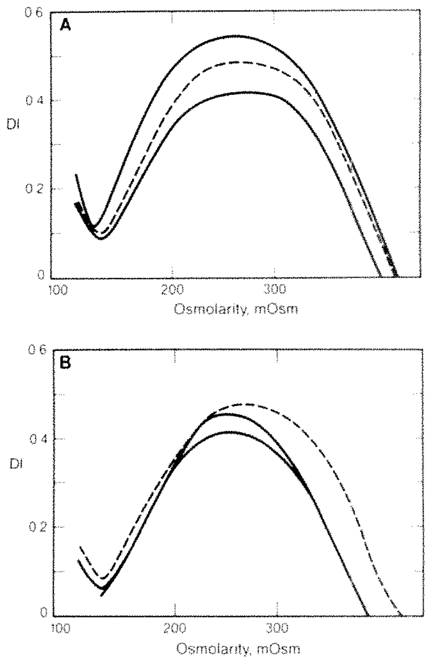

Fig 4. DI as a function of osmolarity measured by ektacytometry. The osmolarity scale is not linear. (A) A rapidly vesiculating blood sample (donor 5). (B) A slowly vesiculating blood sample (donor 4). In each case the upper solid line is the pattern obtained from a fresh sample, and the lower solid line is the pattern obtained after 6 weeks of storage. The dashed line is the pattern of fresh blood from a normal donor analyzed with each sample to serve as a reference point.

Vesiculation During Storage Does Not Correlate With ATP Levels

It is likely that membrane vesiculation and the subsequent loss of deformability is the elusive "storage lesion" that limits the shelf life of stored blood. Therefore, we followed ATP levels and spectrin oxidation during storage to determine whether changes in either of these parameters correlated with vesiculation. Although energy depletion induces vesiculation of erythrocytes in vitro[27] vesiculation during storage did not appear to be related primarily to decreased levels of

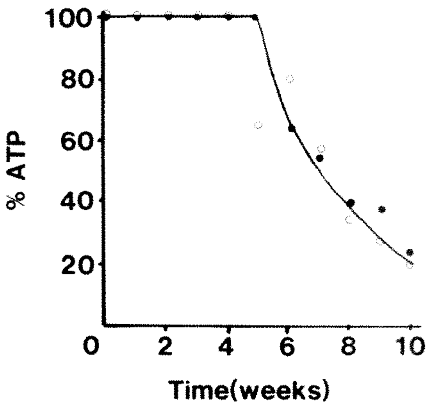

Fig 5. Percentage of the initial ATP level *v* time for donor 1, the most actively vesiculating sample, on two separate occasions. Similar results were obtained on blood from the other donors.

ATP. In most units, vesiculation during storage began immediately (Fig 3) and was active while cellular ATP levels were high (Fig 5).

Vesiculation During Storage Correlates With Spectrin Oxidation

Although vesiculation of blood stored in CDP-A was unrelated to ATP depletion, thiol-disulfide chromatography performed on stored blood samples provided evidence that vesiculation and spectrin oxidation are related. Representative electrophoretic patterns of oxidized chromatography fractions are shown in Fig 6A. It can be seen that the spectrin band of the sample stored for 6 weeks is considerably more dense than that seen in a fresh sample from the same donor. In Fig 6B the change in the spectrin:band 7 ratio with time for 4 units is shown. The shape of the curve for each unit is remarkably similar to its corresponding curve in Fig 3. In Fig 7, the spectrin:band 7 ratio is plotted against the percentage of AChE release (vesiculation). Not only is there excellent correlation between the extent of spectrin oxidation and the extent of vesiculation ($r = .92$), but points derived from all units appear to fall on the same line.

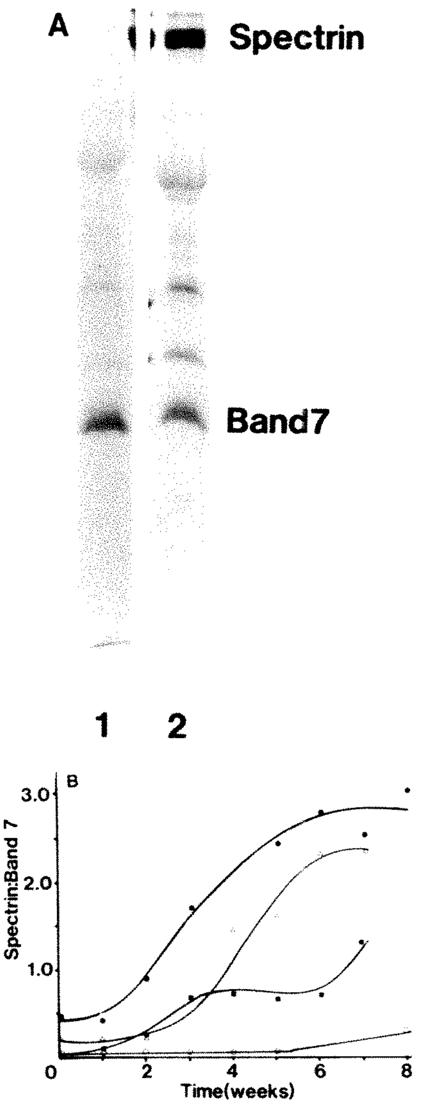

Fig 6. (A) Representative SDS gels of oxidized fractions from donor 1 obtained during thiol-disulfide exchange chromatography. Lane 1, fresh sample. Lane 2, sample after 6 weeks' storage. (B) Spectrin:Band 7 ratio as a function of time. Donor 1, ●—●; donor 2, △—△; Donor 3, ■—■; donor 4, ○—○. The symbols for each donor correspond to those used in Fig 3.

DISCUSSION

The occurrence of membrane vesiculation and the resultant formation of poorly deformable spherocytes is likely to be the cause of the disappointingly short shelf life of stored blood. We have shown that during storage, oxidation of spectrin is occurring and that this process correlates well with vesiculation. This observation complements those of Wolfe et al who found that during storage a reducing agent correctable defect in spectrin:actin:protein 4.1 interaction occurs.[11] Becker et al demonstrated that oxidation of as few as one spectrin sulfhydryl group can produce this defect in a purified protein system and that oxidation of two sulfhydryl groups produces structural alterations in spectrin binding to inside-out vesicles.[28] Thiol-disulfide chromatography detects only severely oxidized proteins that may represent only a small fraction of those that have undergone some degree of oxidative damage. Although mildly oxidized spectrin may be involved in the vesiculation process, vesiculation clearly correlates with the amount of severely oxidized spectrin.

The exact mechanism by which vesiculation occurs is unknown, but it is conceivable that, as spectrin is oxidatively damaged in a small area of the membrane, localized spectrin-lipid interactions are disrupted, and the affected membrane pinches off as a spectrin-depleted free vesicle. Many such events occurring during the storage period would ultimately produce a rigid spherocyte. Why metabolically intact RBC under storage conditions are more prone to vesiculate and become spherocytes than cells in vivo is unknown. Perhaps in vivo the red cell itself has mechanisms for repair of oxidative damage to membrane proteins that are not functional during storage at 4°C, eg, ATP-dependent proteases,[29] or perhaps the reticuloendothelial system is capable of removing damaged membrane in a manner that is less

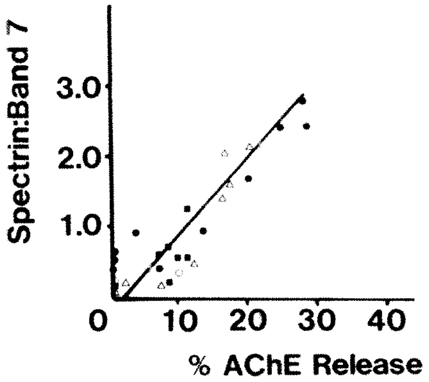

Fig 7. Spectrin:band 7 ratio as a function of the percentage of AChE release. The different symbols represent points derived from different units of blood and correspond to those used in Figs 3 and 6 ($r = .92$).

disruptive to the cell than vesiculation. In any case, our observations support the concept that oxidation of membrane components is involved in the storage lesion and suggest that the use of antioxidants or agents that inhibit vesiculation might be useful for prolonging the shelf life or improving the quality of blood used for transfusion.

ACKNOWLEDGMENT

The authors wish to thank Dr William C. Mentzer at San Francisco General Hospital for allowing us to measure ATP levels on his Picolite (Packard) and Patricia Ross for preparing the manuscript.

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Identification of an Erythropoietin-Sensitive Cell Line

By Donald R. Branch, Jean-Michel Turc, and Larry J. Guilbert

The murine lymphoblastic cell line DA-1 has been characterized as dependent upon both interleukin-3 (IL-3, multicolony-stimulating factor [multi-CSF]) and granulocyte-macrophage colony-stimulating factor (GM-CSF) for survival and growth. Here we demonstrate that it is responsive to a third hematopoietic factor, the erythroid-specific hormone, erythropoietin (Epo). DA-1 cells are stimulated to proliferate by partly purified natural murine and human Epo, and pure recombinant human Epo. Antibody to Epo specifically blocks Epo-stimulated growth. Maximal growth stimulated by Epo and GM-CSF is similar, and considerably less than that stimulated by multi-CSF. Proliferation stimulated by Epo and GM-CSF is transient, decreasing within 24 to 48 hours of exposure. However, Epo acts cooperatively with GM-CSF to sustain proliferation. With or without GM-CSF, no obvious erythroid differentiation of DA-1 cells occurs after exposure to Epo for up to 72 hours. This is the first report of a growth factor-dependent cell line also responsive to Epo for survival and growth. The availability of this cell line model should greatly facilitate biochemical analysis of the mechanism of Epo growth-stimulating action.

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ERYTHROPOIETIN (Epo) is the primary hormone regulating red blood cell formation.[1] It has been purified to homogeniety from human urine, characterized as a single sialoglycopeptide of molecular weight (MW) 35,000, and has been molecularly cloned and expressed.[2-4] The factor targets the committed members of the erythroid differentiation lineage.[5,6] It acts cooperatively with multi-colony–stimulating factor (multi-CSF) and granulocyte-macrophage colony–stimulating factor (GM–CSF) on the least mature of these cells, the burst-forming-unit–erythroid (BFU–E).[1] The response of primary cells is growth-accompanied by differentiation to a nongrowing state.[1]

Epo-responsive progenitor cells occur at less than 1% of normal marrow cells and are maturationally heterogeneous.[1] Thus, they are not a suitable population for biochemical analysis of Epo stimulation. Large increases in the numbers and frequency of these cells have been achieved by exploiting transient erythroblastosis induced by either infection by erythroleukemia viruses[7] or by chemically-induced anemias.[8] However, Epo-responsive cells still constitute a minority of the resulting hematopoietic tissue and, even after purification, probably are not entirely free of contaminating cells. These difficulties in obtaining suitable target populations have hampered molecular-level studies of the response of cells to Epo.

One solution to this problem would be a cell line dependent upon Epo for survival, growth, or differentiation. Although autonomously growing erythroleukemia cell lines have been reported to respond to Epo with increased cloning efficiency and partial differentiation,[9,10] completely dependent lines have not been documented. Here we characterize a growth factor-dependent cell line that proliferates in response to Epo. We also show that the response to Epo, like that of primary cells, is transitory.

MATERIALS AND METHODS

Cells

The DA-1 cell line, first described by Ihle as lymphoblastoid and dependent upon interleukin-3 (IL-3, multi-CSF) for growth,[11] and more recently reported to be also responsive to GM–CSF[12,13] was obtained from Dr K.C. Lee (University of Alberta, Edmonton). The cells were routinely cultured in suspension in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY) containing fetal calf serum (FCS, 10% vol/vol, Flow Laboratories, McLean, VA) plus medium conditioned by WEHI-3 cells (WEHI-3CM, 20% vol/vol). The murine myelomonocytic cell line WEHI-3 (American Type Tissue Culture Collection, Rockville, MD) was routinely cultured in suspension in IMDM containing FCS (15% vol/vol) and 10^{-5} mol/L 2-mercaptoethanol.

Hematopoietic Growth Factor (HGF) Preparations

WEHI-3 CM was harvested from high density cultures ($>10^6$/mL), sterilized by filtration (Millipore, 0.22 µm), and stored at 4°C. WEHI-3 CM contains high levels of multi-CSF and low levels of CSF-1.[14] Pure recombinant murine GM–CSF (rGM–CSF), a gift from Dr R.J. Tushinski, Immunex Corp, Seattle, was prepared as a 100-µg/mL solution in 0.1% trifluoroacetic acid, 48% acetonitrile, and 0.01 mol/L Tris, and stored in liquid N_2. Aliquots were diluted to 1 µg/mL in 0.01% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for use as a working stock. Pure recombinant human erythropoietin (rEpo), a gift from Dr E. Fritsch, Genetics Institute, Boston, was produced by transfecting Chinese hamster ovary (CHO) cells with an expression plasmid containing a human Epo insert,[3] then was purified to homogeniety. It was prepared as a 100 U/mL stock in IMDM containing FCS (5% vol/vol). Purified human urinary Epo (150 U/mL, lot 78-79P) was purchased from the British Columbia Cancer Institute, Vancouver, BC. Purified murine Epo (24 U/mL, lot 10405) was obtained from HyClone Laboratories, Logan, UT. Recombinant murine multi-CSF (rMulti-CSF), a gift from Dr T. Mosmann, DNAX Corp, Palo Alto, CA, was used as an unpurified supernatant from plasmid-transfected COS cells.

Assays of DA-1 Proliferation

Assays were carried out in IMDM containing FCS (5% vol/vol) and incubated at 37°C in a fully humidified atmosphere containing 5% CO_2 in air.

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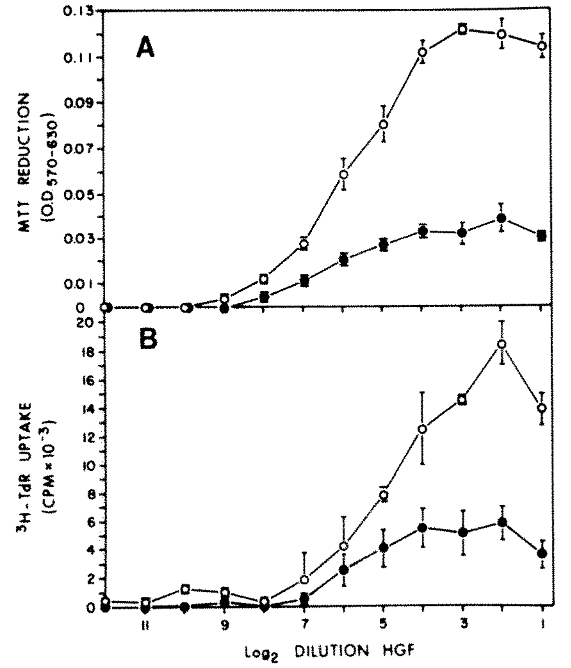

Fig 1. DA-1 cell proliferation as a function of concentration of WEHI-3 CM ○ and human urinary Epo ●. Growth after 24 hours of culture in microtiter plates as measured by (A) MTT reduction and (B) ^{3}H-TdR uptake (see methods for details). Each point is the mean of three replicates, bars denote standard deviation. Abscissa: Log$_2$ of the dilution of undiluted WEHI-3 CM and Epo at 75 U/mL.

Microtiter plate cultures. Growth factors were diluted by serial doublings in the wells of 96-well flat-bottom microtiter plates (Linbro, Flow Labs) in IMDM containing FCS (5% vol/vol). Each well contained 10^4 DA-1 cells in a final volume of 50 μL. The plates were incubated from 24 to 48 hours. Proliferation was assessed in terms of: [a] Tritiated thymidine (^{3}H-TdR, New England Nuclear, Boston) uptake as a measure of the number of cells in cycle at any time. Briefly, 1 μCi ^{3}H-TdR supplemented with 0.24 mol/L 5-fluoro-2'deoxyuridine (Calbiochem, La Jolla, CA) and 0.4 mol/L 2-deoxyinosine (Sigma Chemical, St. Louis) was added to each well. After four hours the cells were harvested onto absorbant glass fiber paper and counted for radioactivity using a scintillation counter. [b] Reduction of the tetrazolium dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma). MTT reduction is a measure of the metabolic activity of the population and is proportional to the number of viable cells.[13,15] The 50 μL microcultures were incubated with 10 μL of 1 mg/mL MTT for four hours, the insoluble blue reaction product dissolved in 0.04 mol/L HCl in isopropanol, and the optical density (OD) measured with a dual-beam multiplate reader (Dynatec MR 600, Dynatech Instruments, Torrance, CA) using test and reference wavelengths of 570 and 630 nm respectively. Data are presented as net OD, ie, experimental minus control, where control is medium with cells but without growth factor.

Suspension cultures. DA-1 cells (10^5/mL) from cultures growing at log-phase in WEHI-3 CM, were thoroughly washed in PBS and cultured in upright T25 flasks (CanLab, Mississauga, Ontario). Individual cultures contained either no growth factor or combinations of recombinant factors at the concentrations indicated. Aliquots were withdrawn at intervals, the total cell number determined by particle counting (Coulter Model ZBI, Coulter Electronics, Hialeah, FL), and per cent viable cells estimated by trypan blue exclusion. Parallel aliquots were plated into 96-well microtiter dishes at 50 μL/well for assessment of capacity to reduce MTT.

Antibody Inhibition of Erythropoietin Response

The rabbit antihuman Epo preparation (anti-Epo) used in these experiments was a gift from Dr R. Sasaki, Kyoto University, Kyoto, Japan, and has been previously described.[16] One mL anti-Epo neutralizes approximately 100 U of Epo. Anti-Epo (or normal rabbit serum [NRS] in controls) was incubated at a 1:2 dilution with rEpo (50 U/mL), rMulti-CSF (1:200 dilution of COS supernatant), rGM-CSF (10 ng/mL) or WEHI-3 CM (50% vol/vol) at 37°C for one hour. After incubation, the mixtures were assessed for DA-1 growth-stimulating activity using the 24-hour microculture assay with MTT reduction as the readout.

Hemoglobin Assay

The hemoglobin content of DA-1 cells was quantitated using a modified spectrophotometric method of Clarke et al.[17] Briefly, after 72 hours of incubation with rMulti-CSF, rEpo, or rGM-CSF, cells (2 to 3 × 10^5) obtained from the suspension cultures above, were washed with PBS, pelleted, lysed in 75 μL distilled, deionized water, and incubated in the dark at 20°C for 20 minutes with 425 μL of a solution containing equal volumes of 0.03 mol/L 3,3',5,5'-tetramethyl benzidine (Sigma) in 90% acetic acid and freshly prepared 1% H_2O_2. The absorbance at 515 nm of undiluted sample was measured using a dual-beam spectrophotometer (Perkin-Elmer, Model 124), and quantitated using murine hemoglobin standards that covered the linear response range of 150 to 400 ng per sample. The standards contained lysate from DA-1 cells, grown in WEHI-3 CM, equivalent in cells per sample to the Epo-stimulated samples.

Table 1. Effect of Anti-Erythropoietin on the Proliferation of DA-1 Cells

HGF	MTT Reduction* of Cultures Containing HGF† Plus: NRS‡	Anti-Epo§	Inhibition of Growth‖
WEHI-3 CM	0.136 ± .003	0.151 ± .038	−11% (NS)
rMulti-CSF	0.212 ± .003	0.212 ± .004	0 (NS)
rGM-CSF	0.080 ± .003	0.080 ± .003	0 (NS)
rEpo	0.043 ± .003	0.003 ± .001	93% ($P < .001$)

*Proportional to the number of viable cells (see methods), mean optical density of triplicate samples ± SD.

†Hematopoietic growth factor, used at levels to give maximum possible response.

‡Normal rabbit serum

§Rabbit anti-erythropoietin

‖[OD(NRS) − OD(anti-Epo)] × 100/OD(NRS); significance determined by student's *t* test, NS = $P > .05$

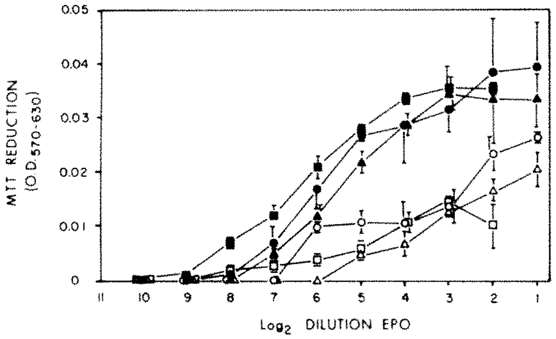

Fig 2. Erythropoietin-stimulated growth of DA-1 cells as a function of Epo concentration and source, and time of culture. Growth measured as described in methods for microtiter plate cultures. Each point represents the mean of three replicates, bars denote the standard deviation. Ordinate: Optical density of MTT reduction product (proportional to viable cell number). Abscissa: Log_2 of dilutions of: ○, pure human rEpo stock of 25 U/mL; □, natural murine Epo stock of 24 U/mL; △, natural human urinary Epo stock of 19 U/mL. Closed symbols indicate number of viable cells as measured by reduction of MTT at 24 hours of culture; open symbols at 48 hours of culture.

RESULTS

DA-1 Response to Epo

As measured by MTT reduction after 24 hours of incubation, the number of viable cells in cultures containing Epo was higher than in controls cultured without hematopoietic growth factor (HGF) (Fig 1A). This increase in the number of viable cells was due to stimulation of proliferation, shown by parallel increases in uptake of ^{3}H-TdR (Fig 1B). Since the urinary Epo preparation could be contaminated with other HGFs active on mouse cells, the experiment was repeated using other Epo preparations, including pure rEpo. The results indicate that DA-1 cells respond to rEpo (Table 1; Fig 2), and that all Epo preparations stimulate growth to the same degree (Fig 2).

Inhibition of DA-1 Proliferation by Anti-Epo Antiserum

Data in Table 1 indicate that anti-Epo completely inhibits the growth-stimulating activity of Epo but not that of WEHI-3 CM, rMulti-CSF, or rGM-CSF. Both these and the above results confirm that Epo alone stimulates proliferation of DA-1 cells. Furthermore, the response is always lower than the maximal response observed with multi-CSF.

Transient Nature of the Response to Epo

The proliferative response of DA-1 cells to all preparations of Epo was greater at 24 hours than at 48 hours (Fig 2); thus, the response appeared to be transient. The kinetics of the response were examined in more detail in suspension cultures of rEpo, rGM-CSF, and rMulti-CSF using two criteria for growth: viable cell counts and capacity to reduce MTT (Fig 3). In the absence of HGF, the cells die within 24 hours. With Epo or GM-CSF alone, maximal numbers of viable

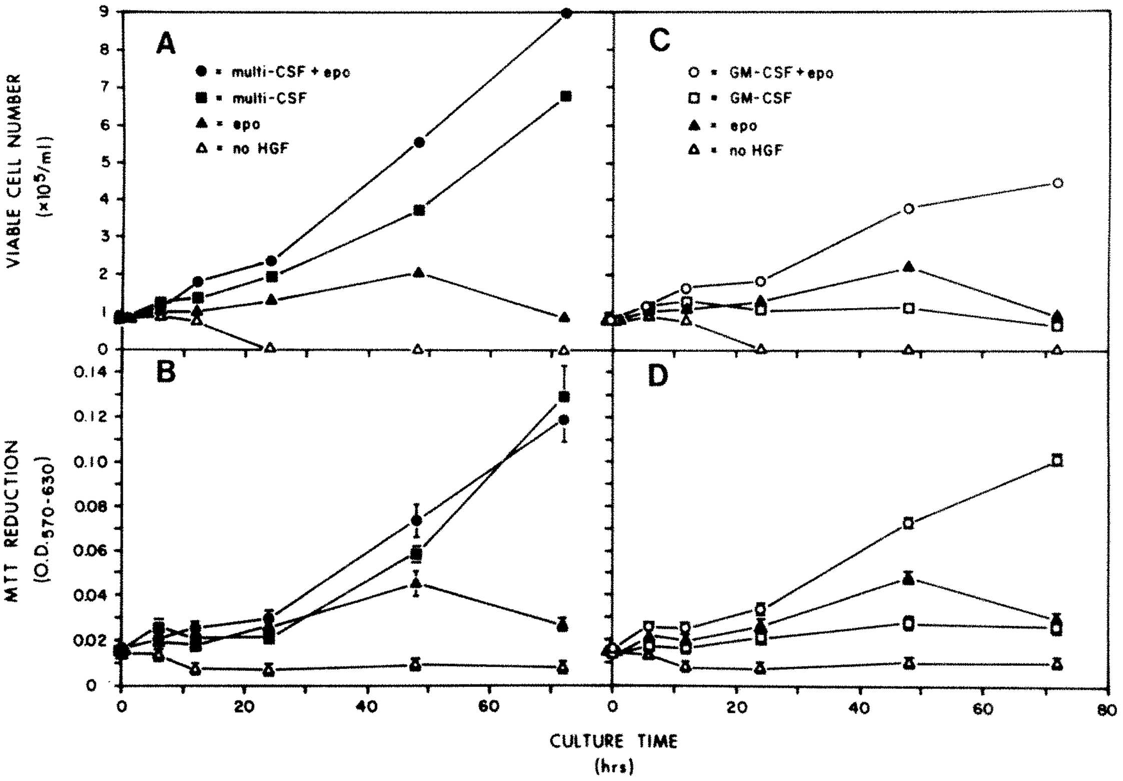

Fig 3. DA-1 cell growth stimulated by Epo, multi-CSF, and GM-CSF from recombinant sources. Individual cultures contain factors as noted. All factors present at maximally stimulating levels: rEpo, 10 U/mL; rMulti-CSF, a 1:2500 dilution of COS supernatant; rGM-CSF, 50 ng/mL. Ordinate: Panels A and C, Viable cell count; panels B and D, optical density of MTT reduction product from a constant volume (50 μL) of cell suspension (see methods). Abscissa: time of culture.

cells were seen at 48 hours, then declined. Using fresh DA-1 cells with MTT reduction as the readout, supernatants collected from the above cultures showed no differences in growth-stimulating activity when compared to zero-time controls (data not shown). Thus, the transient growth was not due to loss of Epo or GM–CSF from the cultures after 48 hours. In contrast to their actions alone, the combination of Epo plus GM–CSF sustained DA-1 proliferation (Fig 3C, D). However, Epo had little effect on the sustained growth stimulated by multi-CSF (Fig 3A, B). These results show that Epo cannot convert sustained growth to transient growth, and that Epo acts with GM–CSF in almost complete synergy to sustain growth beyond the first 48 hours of culture.

Erythroid Differentiation of DA-1 Cells

Obvious erythroid morphologies were not observed in cytospin preparations of DA-1 populations after 72 hours of culture with Epo (data not shown). Furthermore, the red color of hemoglobin was not apparent in centrifuged pellets, and these cells contained less than 150 ng hemoglobin per 200,000 cells ($<$0.75 pg/cell) compared to 10 to 15 pg per BFU–E derived erythroblast.[17] Thus, DA-1 cells are not strongly induced to erythroid differentiation by Epo.

DISCUSSION

We have shown that pure recombinant erythropoietin (rEpo) is able to stimulate the growth of the lymphoblastoid cell line DA-1 and that antibody to Epo specifically blocks this response. This is the first report of a cell line whose growth and survival can be mediated by Epo alone. This observation should facilitate the development of more convenient in vitro models for molecular studies of Epo action.

Whether Epo acts strictly as a growth factor on DA-1 cells or is also able to induce differentiation remains to be determined with certainty. Although no hemoglobin was detected in Epo-treated cells, the assay for hemoglobin has a sensitivity limit of approximately 7×10^6 molecules per cell. Also, our results do not preclude more subtle differentiated properties that may be linked to loss of growth response.

Regardless of whether Epo (or GM–CSF) induces differentiation, it is clear that they are able to act in synergy to sustain the growth of DA-1 cells. Whether multi-CSF at lower concentrations is also a synergist, and whether GM–CSF or multi-CSF are able to increase the sensitivity of DA-1 cells to Epo is under investigation.

A comparison of concentrations of Epo necessary for half maximal stimuation of growth shows the DA-1 response (about 0.3 to 0.4 U/mL, Fig 2) to be much nearer the immature erythroid progenitor BFU–E (about 0.3 U/mL[5]) than the relatively mature progenitor CFU–E (about 0.03 U/mL[18]). Although DA-1 cells have been categorized as "lymphoblastic," their phenotype (Thyl$^-$, Ly5$^+$, H-11$^+$, Ia$^-$, 20 αSDH$^+$) is not incompatible with "immature erythroid."[11] Indeed, their growth factor responsiveness (multi-CSF, GM–CSF, Epo) and transient responsiveness to Epo also places them at a BFU-E stage.[1] Thus, by these criteria, DA-1 cells would appear to be best described as a BFU-E transformed to a permanently growing undifferentiated state.

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CORRESPONDENCE

CLINICAL FEATURES IN LARGE GRANULAR LYMPHOCYTIC LEUKEMIA

To the Editor:

The recent article by Chan et al[1] attempts to delineate two major subtypes of large granular lymphocytic (LGL) leukemia,[2] ie, patients with CD3+ LGL *v* patients with CD3− LGL. In their series, CD3+ LGL leukemia patients invariably had neutropenia and frequently had rheumatoid arthritis, whereas the patients with CD3− LGL proliferations did not have these features. These results should be interpreted with caution, since only two CD3− LGL leukemia patients were studied. We have evaluated 38 patients with this disease; six had CD3− LGL proliferations. No apparant clinical differences were observed between patients with CD3+ LGL *v* those with CD3− LGL. Specifically, neutropenia and rheumatoid arthritis occurred in some of the CD3− LGL leukemia patients; conversely, some patients with CD3+ LGL leukemia were not neutropenic. Thus it would appear that the clinical disease is similar in patients with proliferations of either phenotypic subset of LGL. This is substantiated by the recent finding of a clonal cytogenetic abnormality in a patient with CD3− LGL proliferation, showing that this disorder is also a neoplastic process.[3] LGL leukemia, therefore, results from a clonal proliferation of either CD3+ or CD3− LGL that may be associated with rheumatoid arthritis[4] or hematologic abnormalities such as chronic neutropenia,[2] pure red cell aplasia,[5] or adult-onset cyclic neutropenia.[6,7]

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Loughran TP, Hammond WP: Adult onset cyclic neutropenia is a benign neoplasm associated with clonal proliferation of large granular lymphocytes. J Exp Med 164:2089, 1986

To the Editor:

Drs Loughran and Starkebaum are correct in pointing out that one should be cautious in drawing conclusions from clinical features observed in a limited number of patients. We have in fact stated in our manuscript[1] that "More Type B patients have to be studied, however, to determine whether the clinical features presented here are consistently observed in this type of LGL lymphocytosis." We have now studied a total of four type B (CD3−) patients[2] and one of them did have associated neutropenia but rheumatoid arthritis and autoantibodies have not been observed yet. A large multinational study on LGL lymphocytosis initiated by Dr Pandolfi is now in progress and it may help to clarify the similarity and differences in clinical features between these two types of patients.

While there is close morphologic similarity and also some overlapping clinical manifestations between Type A (CD3+) and Type B (CD3−) LGL lymphocytosis, it is quite clear that CD3+ LGL are derived from the T cell lineage, whereas CD3− LGL have little indication of T cell differentiation.[3,4] This difference in histogenesis and/or differentiation would clearly warrant their separation into two subtypes.

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ANNOUNCEMENTS

AN OPEN INTERNATIONAL DISCUSSION SYMPOSIUM
"NEW STRATEGIES IN THE MANAGEMENT OF THROMBOTIC DISORDERS"

November 12-14, 1987
Loyola University Medical Center, Maywood, IL

DISCUSSANTS (PROPOSED)

N. Bang, *Indianapolis, IN*
K. Breddin, *Frankfurt, W Germany*
G.V.R. Born, *London, England*
J.P. Caen, *Paris, France*
J. Caprini, *Chicago, IL*
J. Choay, *Paris, France*
U. Cornelli, *Milan, Italy*
J.F. Davidson, *Glasgow, UK*
J. Fareed, *Chicago, IL*
M. Gent, *Hamilton, Ontario*
V. Gurewich, *Boston, MA*
F. Leja, *Chicago, IL*
V.J. Kakkar, *London, England*
E.F. Mammen, *Detroit, MI*
H.L. Messmore, *Chicago, IL*
J. O'Brien, *Portsmouth, UK*
M. Samama, *Paris, France*
P. Scanlon, *Chicago, IL*
J. Tobelem, *Paris, France*
O.N. Ulutin, *Istanbul, Turkey*
W.H. Wehrmacher, *Chicago, IL*

Topics: Heparin and its derivatives; Ticlopidine—newer antiplatelet drugs; T-PA and related thrombolytic agents; Defibrotide and related drugs; viscosity modulators; biotechnology-based products; polytherapy of thrombotic disorders; mechanical devices; physical devices; and target-specific agents.

This is an open symposium and all interested individuals are invited to attend. The program will be available in October and will be forwarded to those interested via circulars. No registration fee is required from the participants; however, the registration is limited to the first 200. *For further information, contact:* Dr J. Fareed, Loyola University Medical Center, 2160 S First Ave, Maywood, IL 60153. Telephone: (312) 531-3256.

THE FIRST INTERNATIONAL SYMPOSIUM ON THE THALASSEMIAS IN CHINA

October 12–15, 1987
Shanghai, Peoples Republic of China

Scientific Organizers: Dr Titus H.J. Huisman, *Augusta, GA*
Dr Clark Lum, *Bethesda, MD*
Dr Zeng Yi-tao, *Shanghai, Peoples Republic of China*

The objective of this conference is to bring together experts from different countries to meet with their Chinese colleagues to stimulate further research in these diseases and to promote and establish scientific contacts.

For further information, contact: Dr Titus H.J. Huisman, Medical College of Georgia, Augusta, GA 30912-3331, or write to the official travel agent: Amerasia Travel Agency, Inc, 3000 Connecticut Ave, NW, Suite 238, Washington, DC 20008.

THE REGULATION OF HEMATOPOIESIS

August 29–30, 1987
Tianjing, Peoples Repulic of China

This workshop will be held under the Chairmanship of Dr Chien Wen-Chieh at (and sponsored by) the Institute of Hematology, Chinese Academy of Medical Sciences. The topics to be addressed are: Molecular and cellular regulation of hematopoietic progenitor cells, and clinical and preclinical studies of experimental hematology and leukemia. Abstracts must be submitted by June 30, 1987, to:

Dr Song Zengxuan
Institute of Hematology
Chinese Academy of Medical Sciences
288 Nanking Rd
Tianjing, Peoples Republic of China

For more information, please contact: Dr Li Lu, Indiana University, Department of Medicine, Clinical Building, Room 379, Indianapolis, IN 46223. Telephone: (317) 274-3589.

BONE MARROW TRANSPLANTATION

August 31, 1987
Tianjing, Peoples Republic of China

This workshop will be held at (and sponsored by) the Institute of Hematology, Chinese Academy of Medical Sciences. Presentations on various aspects of bone marrow transplantation will be made by: Fred Appelbaum, C. Dean Buckner, Guido Lucarelli, Richard J. O'Reilly, Robertson Parkman, and George Santos.

For more information, please contact: Dr Clark K. Lum, National Institutes of Health, WW355, Division of Research Grants, Bethesda, MD 20892. Telephone: (301) 496-7508. *For travel and visa arrangements, please contact:* Amerasia Travel Agency, Inc, 3000 Connecticut Ave, NW, Suite 238, Washington, DC 20008. Telephone: (202) 232-8877. Telex: 4942109 ATA WSH.

FOURTH TERRY FOX CANCER CONFERENCE ON STEM CELLS AND AUTOLOGOUS BONE MARROW TRANSPLANTATION

September 20–22, 1987
Vancouver, Canada

The objective of this conference is to bring together both basic scientists and clinicians with common interests in hematopoietic stem cells, their regulation, and new ways for their use in therapeutic purposes. The conference will highlight current results with autologous marrow transplantation, new developments in stem cell biology, purging techniques, clinical use of growth factors, and gene transfer.

The program will consist of two full days of presentations by invited speakers plus a poster session for participants. Registration fee: $100 Cdn ($50 for students or trainees). Deadline for receipt of abstracts: August 1, 1987.

For registration and abstract forms, contact: Fourth Terry Fox Cancer Conference Organizing Committee, Terry Fox Laboratory, BC Cancer Research Centre, 601 W 10th Ave, Vancouver, BC, Canada V5Z 1L3. Telephone (604) 877-6070.

ERRATA

In the article by Parker and Gralnick entitled "Identification of Platelet Glycoprotein IIb/IIIa as the Major Binding Site for Released Platelet-von Willebrand Factor," which appeared in the September 1986 issue (Volume 68, pp 732-736), there was an error in Table 1. The error is located in the column head over the right hand columns; the superscript exponent "3" was omitted. The column head should read "10^3 Molecules Anti-vWF Bound per Platelet."

In the article by Blume et al entitled "Total Body Irradiation and High-Dose Etoposide: A New Preparatory Regimen for Bone Marrow Transplantation in Patients With Advanced Hematologic Malignancies," which appeared in the April 1987 issue (Volume 69, pp 1015-1020), there was an error regarding one of the 47 marrow transplant recipients in the study: Patient 183 in Table 1 had been treated with 25 mg etoposide/kg body weight (as described), 1,000 cGy total body irradiation (instead of the reported 750 cGy), and cytosine arabinoside (2 × 5 mg/kg body weight).

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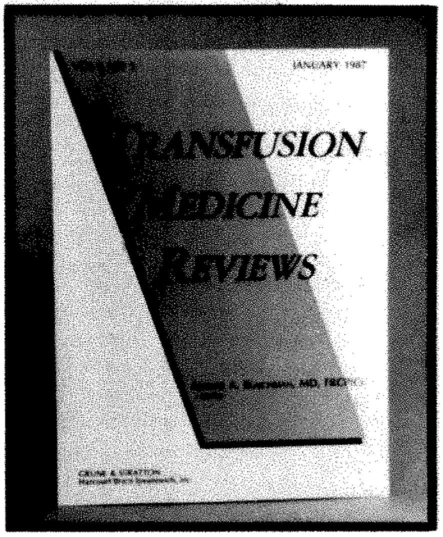

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- Post transfusion non-A, non-B, hepatitis. *Harvey J. Alter*
- The association of blood group antigens with red cell membrane glycoproteins. *David J. Anstee.*
- The effects of red cell antigens and blood transfusion on renal transplantation. *Morris A. Blajchman*
- The role of the hematocrit in bleeding. *Bernard Boneu (with F. Fernandez)*
- Complications associated with apheresis procedures. *Marc Boogaerts*
- Controversies in the use of Rh immunoglobulin in the prevention of Rh alloimmunization. *J.M. Bowman*
- The significance of complement on the red cell surface. *John J. Freedman*
- The significance of IgG on the red cell surface. *George Garratty*
- The role of platelet glycoproteins in platelet function. *James N. George*
- Putative mechanisms of the Factor VIII bypass activity of Factor IX concentrates. *Alan Giles*
- Transfusion associated AIDS. *Jerome E. Groopman*
- The Factor VIII molecule-new perspectives. *Leon W. Hoyer*
- The use of intravenous IgG in the treatment of immune disorders. *John Kelton*
- The significance of reticuloendothelial function in the immune destruction of blood cells. *John Kelton*
- Immunogenicity of platelet membrane glycoproteins. *Tom Kunicki*
- Treatment of patients with Factor VIII antibodies. *Jeanne M. Lusher*
- Recent Developments in the Kell blood group system. *W. Laurence Marsh*
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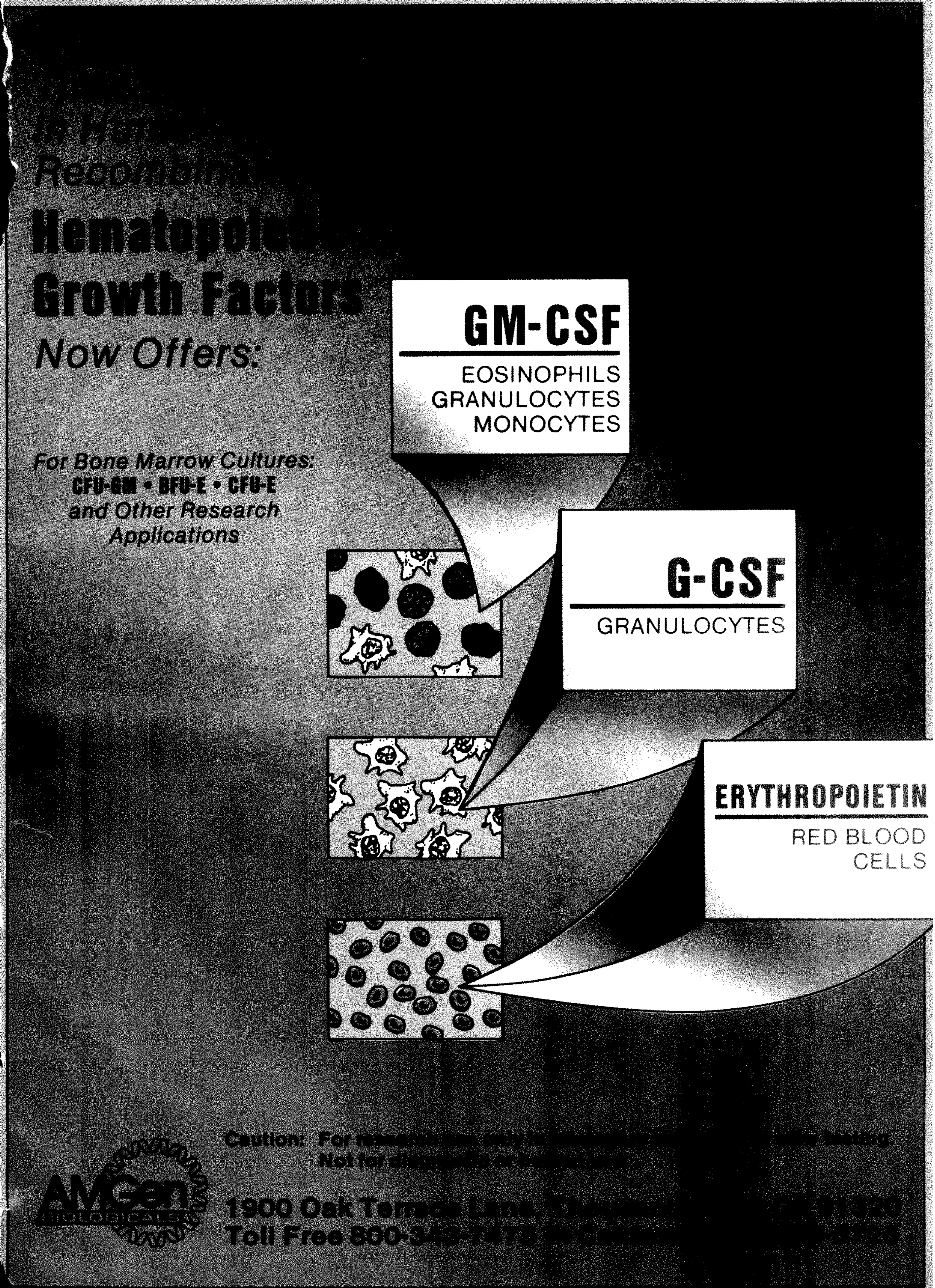

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